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Part III Immunology of Mycoses

Part IV Therapy

INTRODUCTION

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On September 6, 1950, The New York Academy of Sciences[1] published a monograph entitled *Medical Mycology*. At that time interest in this field was growing steadily. Nearly ten years have passed and there is no question that during that period the field of mycology has expanded and gained in importance. Impressive advances have been made not only in the basic fields of epidemiology, immunology, biochemistry, and physiology but also, more recently, in therapy. These contributions, which in some cases were documented in the literature and in others were only discussed when investigators met privately, have prompted this publication.

Many eminent scientists from Indonesia, Mexico, Portugal, France, and many parts of the United States are contributors to this monograph. The list of contributors reads like a *Who's Who in Medical Mycology*. Chester W. Emmons, with the aid of his committee, planned a well balanced publication. Emphasis was put on the fundamental aspects of the field. For example, the importance of phycomycosis, physiological studies on coccidioides and studies on immunology and serology are presented. The newer advances in therapy also are not neglected although an attempt has been made to present them in a concise and critical form.

Tribute should be paid to one scientist who, with his usual modesty, remained in the background, but who made all the preparations and led the groundwork for the conference on which this monograph is based. Emanuel Grunberg of the Division of Mycology of The New York Academy of Sciences, devoted countless hours to the preparation of that conference. Only those who have tried to organize an international conference of such proportions can realize how many difficulties and problems are involved in an undertaking of this kind. In this great task Grunberg was aided and supported by Chester W. Emmons.

The past ten years have seen impressive progress in medical mycology. I am sure that this monograph will contribute significantly to the field of experimental and clinical medicine not only by providing new and valuable information but also by stimulating new and fruitful developments.

Reference

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Part I. General, New, and Experimental Mycoses

SUBCUTANEOUS PHYCOMYCOSIS. A NEW DISEASE FOUND IN INDONESIA

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Subcutaneous phycomycosis is a localized eosinophilic granuloma caused by a phycomycete that invades primarily the fat layer of the subcutaneous tissue Three isolations of a *Basidiobolus* species and one of an unidentified phycomycete have been made in Indonesia

Five cases of subcutaneous phycomycosis have been found in Indonesia, and one case was observed recently in London, England, by W St C Symmers in a Dutch girl who had come from Indonesia Spontaneous healing took place after varying periods of time

The name phycomycosis is used to designate a fungus infection caused by a member of the phycomycetes Its use was proposed in a previous paper (Lie Kian Joe *et al*, 1959) in order to avoid the more restricted connotations of mucormycosis, while the name of coccidioidomycosis is to be retained The name phycomycosis includes infections caused by *Mucor*, *Absidia*, *Rhizopus*, *Basidiobolus*, and similar phycomycetes The term is useful, too, for mycoses in which no culture was obtained, but in which sections of involved tissues revealed the presence of a fungus with a morphology usually associated with a phycomycete

Clinical Characteristics

The disease has been observed only in children varying in age from four to thirteen years The infection begins as a movable subcutaneous nodule that gradually increases in size by peripheral growth to attain extensive proportions covering a large area of the body (FIGURES 1, 2, and 3) The swelling is painless and, when fully developed, may be 3 to 4 cm thick The border with the healthy tissue is demarcated sharply The surface of the swelling is smooth or it may show superficial lobation or nodules Its consistency is firm, but there may be small, scattered soft areas The skin covering the swelling is usually normal but, when the swelling is extensive, the skin is edematous and bluish red in color In one case the skin was pruritic, scaly, and covered with secondary infections from excoriation (FIGURE 2)

The swelling is usually not adherent to the superficial fascia The edge of the swelling often can be lifted somewhat from the underlying layer It forms a firm thick sheet of tissue lying between the corium of the skin and the superficial fascia When surgically removed, it is easily loosened from the overlying and underlying tissue

The swelling grows slowly Growth may be restricted only to one or two areas of the swelling, and growth is often periodic The swelling may progress at one edge while regressing at the opposite edge

The duration of the disease may be from several months to more than four years In long lasting cases the size of the lesion may be considerable Spontaneous healing has been observed in four cases One case died from other causes and in the most recent case the lesion is regressing after administration of oral potassium iodide

There is apparently no site of predilection for the lesion With the exception of one case where three lesions began simultaneously on the scrotum right thigh and left buttock the lesions are solitary but extend to cover large areas One patient had an extensive lesion that started as a small nodule on the thorax This extended gradually to both axillae both upper arms and the upper part

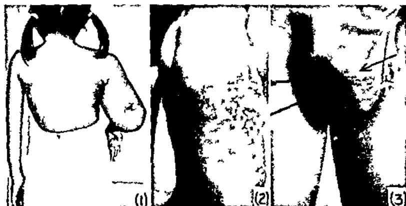


Figure 1 Swelling of the entire right upper arm and shoulder in a girl aged 7 years Scars on the dorsal part of the upper arm and near the axilla are from biopsies (case 3)
Figure 2 Swelling of the entire right thigh and buttock in a boy aged 8 years (case 4) The skin covering the swelling shows secondary infection
Figure 3 Swelling on the right scrotum (*arrows*) of 1 month duration in a boy aged 5 years (case 5)

of the abdomen While these lesions were regressing the swelling extended gradually to the buttocks crossing the flanks before spontaneous healing occurred

TABLE 1 shows the pertinent data of the cases observed

Lymphogenous and hematogenous spread of the disease apparently do not occur The regional lymph glands are not enlarged unless there is secondary bacterial infection

The general condition of the patient is usually good Fever was observed in only one patient who had a secondary infection of the lesion The blood sedimentation rate was high in two of four patients examined There was increased blood eosinophilia in two of four patients studied In one of these two cases it was not known whether worm infection was present but the other case was apparently free from worm parasites and no other cause for the eosinophilia could be found

TABLE 1
SUBCUTANEOUS PHYCOMYCOSIS

Case no	Author	Age	Sex	Location of lesion	Duration of disease	Isolation of fungus	Outcome of disease
1	Lie Kian Joe *et al* (1956)	4	M	Left pectoral region	9 months	*Basidiobolus ranarum* (?)	Spontaneous recovery
2	Lie Kian Joe *et al* (1956)	8	M	Thorax axillae upper arms abdomen flanks, buttocks	More than 4 years	—	Spontaneous recovery
3	Lie Kian Joe *et al* (1960)	7	F	Right upper arm and shoulder	1½ years	Unidentified phycomycete	Spontaneous recovery
4	Tio *et al* (1960)	8	M	Entire right thigh and buttock	1 year	*Basidiobolus* sp	Regressing under potassium iodide treatment
5	Tio *et al* (1960)	5	M	Scrotum right thigh, left buttock	1 month	*Basidiobolus* sp from lesions on thigh and scrotum	Died from other cause
6	Symmers (1960)	13	F	Interscapular region	More than 9 months	—	Spontaneous recovery

Histopathology

The swelling consists of granulomatous tissue. This granulomatous tissue replaces the subcutaneous fat tissue. Remnants of fat cells may be found within the pathological tissue. Cross and longitudinal sections of large branching hyphae with infrequent septations are seen within the granulomatous tissue. The diameter of the hyphae generally varies from 8 μ to 12 μ. The hyphae occasionally may be smaller, from 5 μ in width, or swollen to 22 μ (FIGURE 13). The walls are dark blue in color in the hematoxylin and eosin-stained slides.

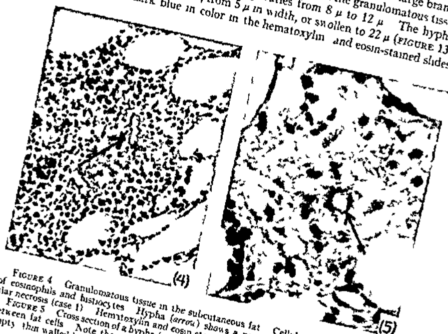


FIGURE 4. Granulomatous tissue in the subcutaneous fat. Cellular infiltration consists of eosinophils and histiocytes. Hypha (*arrow*) shows a narrow layer of eosinophilic, granular necrosis (case 1). Hematoxylin and eosin stain. ×340.
FIGURE 5. Cross section of a hypha (*arrow*) found in an offshoot of the granulomatous tissue between fat cells. Note the narrow layer of eosinophilic granular necrosis surrounding the empty thin walled hypha (case 4). Hematoxylin and eosin stain. ×570.

They are often thin and not easily seen but occasionally they may be thick, as is often the case in the large swollen hyphae. Most of the hyphae are empty (FIGURES 5, 6, 8 and 11), probably because of extreme shrinkage of the vacuolated cytoplasm or because the contents have fallen out during the process of staining. Some contain a light purple or light blue staining granular cytoplasm (FIGURE 4). The swollen hyphae occasionally contain dark staining, coarse, granular contents (FIGURE 13). Usually the hyphae have a regular shape, often they do not show protrusions of the wall in longitudinal sections, and usually they are round or oval in cross section. Fragmented, distorted or collapsed hyphae are also seen. These and the swollen ones are probably dead or degenerating hyphae. Branching is seldom seen. Hyphae are usually not numerous in the biopsy specimens, whether taken from the center or from the





FIGURE 6 Longitudinal section of a branching hypha causing a relatively broad area of eosinophilic granular necrosis Note the thin wall of the almost empty hypha Cellular infiltration consists of histiocytes and eosinophils (case 1) Hematoxylin and eosin stain ×504

FIGURE 7 Longitudinal section of a hypha with septa Note eosinophilic granular necrosis cellular infiltration consists of histiocytes and eosinophils (case 3) Hematoxylin and eosin stain ×468





FIGURE 8 Large area of eosinophilic, granular necrosis around empty hyphae (*arrow*) within aggregations of histiocytes (case 3) Hematoxylin and eosin stain ×112

FIGURE 9 Branching hypha with thin walls surrounded by epithelioid histiocytes Note palisading of histiocytes and eosinophilic, granular necrosis along the hypha (case 3) Hematoxylin and eosin stain ×340



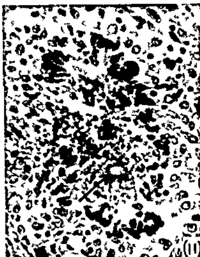


FIGURE 10 Fragment of hypha phagocytosed by a multinucleated giant cell Note absence of eos nophilic granular necros s (case 2) Hematoxylin and eos n stain ×504

FIGURE 11 Empty hypha (*arrow*) w th a th n wall surrounded by ep thelioid h stiocytes and mult nucleated giant cells Marked eos nophil c granular necros s with beginning of abscess format on Pal sad ng of histiocytes (case 3) Hematoxylin and eos n slide ×340

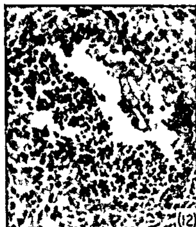




FIGURE 12 M croabscess conta ning eos nophils neutrophi s and a thick wa led hypha Absence of eos nophil c granular necrosis around the hypha (case 1) Hematoxylin and eos n sta n ×259

FIGURE 13 Swollen hypha w th coarse granular content and th ck wall found n a m cro abscess Note absence of eos noph c granular necros s (case 2) Hematoxyl n and eos n stain ×570

peripheral part of the lesion Usually only a few hyphae are found in each slide In some biopsy specimens no hyphae have been found The hyphae usually occur singly, not in groups

The granulomatous reaction accompanying these hyphae is characterized by the presence of numerous eosinophils, histiocytes, pseudotubercles with foreign body giant cells, a number of small abscesses, and small necrotic areas In addition, fibroblasts and fibrous tissue may be present

A characteristic feature of the granulomatous tissue is the presence of numerous eosinophils These cells are found throughout the whole pathologic tissue In certain areas they are present in such large numbers that the area is stained red in hematoxylin- and eosin stained slides There is a tendency for the eosinophils to accumulate about the hyphae, but actually they are found everywhere in the pathologic tissue

The most characteristic feature of the granulomatous tissue is the presence of a peculiar tissue necrosis occurring around the hyphae The necrotic tissue stains red in the hematoxylin and eosin stained slides, and it has a smudgy, granular appearance We call this eosinophilic granular necrosis This type of necrosis is unique, since it is not found in other mycotic infections

The necrotic layer surrounding the hyphae is usually not broad, varying in width from 3 μ to 15 μ (FIGURES 4 and 5) Sometimes necrosis may be more marked, resulting in a broader eosinophilic, granular zone (FIGURE 6) Occasionally, necrosis is so marked that relatively large areas surrounding the hyphae are stained red (FIGURE 8) However, not all hyphae are surrounded by necrotic tissue Hyphae not associated with necrosis are often distorted or fragmented or swollen They are probably dead or degenerated, and are found frequently within foreign body giant cells or in microabscesses Apparently, active hyphae cause necrosis, and dead or inactive hyphae do not It is assumed, therefore, that this peculiar necrosis is caused by specific metabolic products from the hyphae

The extent of the necrosis depends probably not only on the amount of the metabolic products found, but also on many other factors such as the speed with which the metabolic products diffuse into the tissue, the speed with which the body gets rid of them the resistance of the tissue involved, and other unknown factors Relatively large areas of necrosis have been found within aggregations of epithelioid histiocytes and in areas with cellular infiltration Apparently, only narrow ring necrosis is seen in fibrous tissue In one case, invasion of the fungus into the muscle tissue occurred, resulting in widespread hyalin degeneration and leading to necrosis of the muscle fibers (Lie Kian Joe *et al*, 1960) Despite this, the fungus did not grow deeper into the muscles but remained superficial in a localized area No eosinophilic, granular necrosis is found in the muscle fibers

Microabscesses contain neutrophils and eosinophils In some, neutrophils predominate, in others, eosinophils and occasionally pure eosinophilic abscesses are seen Microabscesses often contain a hypha (FIGURE 12) Many of these hyphae are probably dead, but hyphae showing an eosinophilic layer of necrosis may also be found within microabscesses These microabscesses are usually not numerous They are found frequently within aggregations of epithelioid histiocytes resulting in suppurating pseudotubercles (FIGURES 11 and 14)

They are also found in other areas of the granulomatous tissue, but apparently they are not present in fibrous tissue The microabscesses are formed by suppuration of the larger necrotic areas Partly suppurated necrotic areas or remnants of the necrotic material at the periphery of the abscesses are often seen

Palisading of histiocytes around hyphae, necrotic areas, and microabscesses is seen and is characteristic (FIGURES 9, 11 and 14) Multinucleated giant cells may contain fragments of hyphae (FIGURE 10) They may also contain eosinophilic granular material that is, apparently, phagocytosed necrotic tissue

In certain areas of the granulomatous tissue, cellular infiltration predominates in others, pseudotubercles and multinucleated giant cells, and in still

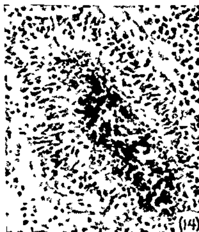


FIGURE 14 Eosinophilic granular necrosis showing suppuration Note palisading of histiocytes (case 3) Hematoxylin and eosin stain ×170

other areas, fibroblasts and fibrous tissue The latter tissue reaction is prob ably a manifestation of the healing process that may be found next to active granulomatous tissue In far advanced healed lesions only fibrous tissue is seen

Hyphae are found chiefly in active granulomatous tissue They are seldom found in areas with fibroblasts and fibrous tissue They are never found outside the granulomatous tissue There is no tendency for the hyphae to grow into the blood vessels which usually show no abnormalities except that many of them are dilated

The periphery of the granulomatous tissue is well separated from the healthy fat tissue In many places offshoots of the granulomatous tissue extend between the fat cells into the healthy tissue These offshoots consists of infiltration with histiocytes and eosinophils Apparently, these offshoots are the routes by which the metabolic products of the fungus are carried away from the lesion In the narrow offshoots no hyphae have been found, but in the broader ones hyphae may be seen (FIGURE 4)

The corium of the skin shows only infiltration with round cells and often a number of eosinophils in the hair follicles around sebaceous glands around the sweat glands and around the blood vessels Hair follicles and sebaceous glands may become atrophic In certain areas invasion of the lower part of the corium by granulomatous tissue and hyphae is seen The epithelium is intact unless secondary bacterial infection is present Parakeratotic areas may be seen in the epithelium covering the lesion

Isolation of the Fungus

A fungus was isolated from four cases From three cases a *Basidiobolus* species was cultured The fungus obtained from the first case was identified as *Basidiobolus ranarum* (Emmons *et al* 1957) Drechsler (1958) who studied this strain is of the opinion that it differs from *B ranarum* as described by Eidam

The two most recent isolations of *Basidiobolus* are being studied currently Another phycomycete was isolated from one patient, but it could not be identified since spores did not develop and the strain soon died

The genus *Basidiobolus* belongs to the family Entomophthoraceae a saprophytic fungus of the intestinal tract of insectivorous animals It has been found in Indonesia in the gastrointestinal contents from frogs (*Bufo melanosticus*) toads (*Rana cancrivora*), gekkoes (*Gecko gekko* and *Hemidactylus renatus*) and chameleons (*Calotus jubatus*) These animals are believed to acquire their infections from insects that feed on the animals' excrements and are in turn eaten by the animals (Levisohn 1927)

Discussion

Subcutaneous phycomycosis is a characteristic disease entity Its chief clinical feature a localized painless subcutaneous swelling that grows gradually to attain extensive proportions and is covered by the intact skin is so typical that one would suspect the disease from clinical examination only

The histological picture of the lesion is also typical It shows a granulomatous reaction characterized by the presence of numerous eosinophils and smudgy eosinophilic granular tissue necrosis around the hyphae The eosinophilic granular necrosis is unique and is not found in other mycotic infections

Marked tissue eosinophilia is also a characteristic feature and is rarely found in fungus infections It was found in man once in a case of phycomycosis of the stomach wall associated with the presence of a large ulcer (Lie Kian Joe and Njo Injo Tjoei Eng 1956) However no eosinophilic granular necrosis around the hyphae was found in this case Moreover the histopathology and the morphology of the fungus were quite unlike the appearance in subcutaneous phycomycosis

Marked tissue eosinophilia was also described by Christiansen (1929) in pigs that showed infection with *Rhizopus* and *Absidia* but here again there was no eosinophilic granular necrosis of the tissue while the clinical picture of the disease was different from that of subcutaneous phycomycosis

Histiocytes are found in large numbers in association with eosinophils In certain areas eosinophils predominate in others histiocytes These two kinds

of cells, along with eosinophilic granular necrosis, form the basic histological change in this disease These changes are probably caused by specific metabolic products of the fungus The concentration of these metabolic products probably influences the occurrence of necrosis or cellular infiltration Around the hyphae the concentration is high, and necrosis is the result, further away, cellular infiltration occurs Palisading of histiocytes around hyphae, necrotic areas, or microabscesses is not infrequent and is characteristic It is marked in case 3 of TABLE 1 Pseudotubercles and giant cells develop from the histiocytes, while the relatively large necrotic areas may suppurate and become microabscesses Later fibroblasts and fibrous tissue are formed

Spread of the fungus into the peripheral tissue occurs along offshoots of the granulomatous tissue These offshoots are assumed to be the routes through which the metabolic products of the fungus drain away from the lesion As the hyphae grow into the offshoots, these become broader in advance of the hyphae

The growth of the lesion is periodic and slow There is probably marked inhibition of the fungal growth in the tissue as compared with rapid growth in culture The growth of the fungus in the tissue is probably associated with destruction of fat cells since the lesion is located chiefly in the layer of the subcutaneous fat The lower part of the corium where there is no fat tissue, is affected only occasionally In one case there was invasion of the fungus into the muscle tissue, causing widespread hyalin degeneration and necrosis of the muscles Despite this the fungus did not grow deeply into the muscle tissue, but remained localized

Branching of the hyphae in the tissue is seldom found Therefore, hyphae are usually not numerous, they occur singly, not in groups This is a marked contrast with the abundant growth of hyphae found in infections with *Rhizopus* and similar phycomycetes

Hyphae often have a thin wall that makes them inconspicuous in the hematoxylin and eosin stained slides In addition, they are often empty, which makes recognition difficult when they are cut transversely The ring of eosinophilic granular necrosis surrounding many of the hyphae is extremely useful for locating them This eosinophilic, granular ring stands out clearly and can be seen under low power magnification When observed under high power, the thin dark blue wall of the often empty hypha can be seen attached to the inner surface of the eosinophilic ring

The hyphae are not distributed evenly throughout the whole pathological tissue In some biopsy specimens, hyphae are readily found, in others they are difficult to find However, the presence of eosinophilic granulomatous tissue is a good clue to follow in making a serious attempt to find them

Hyphae are stained red with the periodic acid Schiff stain, red violet with the Gridley modification, and black with Gomori's silver methanamine stain However hyphae with a thin wall often are stained faintly with the periodic acid Schiff stain and with the Gridley method, while the Gomori method gives better results These staining techniques, however, are not advantageous over the ordinary hematoxylin and eosin stain for detecting hyphae, since the eosinophilic, granular necrosis surrounding many of the hyphae is a good indi-

cator for locating hyphae Isolation is sometimes easy, sometimes more difficult, depending probably on the presence or absence of hyphae in the tissue used for culture

Basidiobolus infection has been described once in a man (Casagrandi, 1931) in whom zygospores were found on the surface of a gastric ulcer This report was not convincing, and cultures were not made Overeem (1915) reported isolating *B ranarum* from fungus granules found in an abscess on the leg of a horse in Java, Indonesia

Histological comparison of the cases from which *Basidiobolus* was isolated and the case from which an unidentified phycomycete was obtained revealed the following differences

Hyphae were more numerous in the case with the unidentified fungus Consequently, there was more eosinophilic, granular tissue necrosis and more marked giant cell formation Palisading of histiocytes was also more marked In addition, there was invasion of the superficial fascia and underlying muscle tissue by the fungus in this case Until more cases are available it is difficult to evaluate the significance of these differences Probably much depends on the area where the biopsy is taken, as well as on the stage of development of the lesion

Administration of potassium iodide in one case still under observation has been followed by a marked regression of the lesion This patient failed to respond to mycostatin, penicillin, chloromycetin, tetracyclin, and griseofulvin Penicillin was given to two other patients without clinical response

We have tried to infect cats dogs, rats, mice, and hamsters by inoculating the fungus into the subcutaneous tissue, but with negative results

The mode of infection in man is not known One would assume that infection occurs through introduction of the fungus into the skin by trauma However, in all cases no history of a trauma at the site of the first lesion could be obtained Moreover, if infection took place through traumatic introduction of the fungus, one would expect the lesion to be located more frequently in parts of the body such as the feet or hands which are exposed most often to trauma

Cases of phycomycosis affecting organs other than the subcutaneous tissue and probably caused by other members of the phycomycetes have been reported in different countries A few cases reported before 1943 in the German and French literature were summarized by Gregory *et al* (1943) Most of these were of pulmonary infection while cases affecting the ears and gastric wall were also reported Infections of the nose and skin were quite uncommon, and the central nervous system was affected least Generalized dissemination of the fungus was observed once

Since 1943 reports of phycomycosis have increased in frequency The disease has been reported chiefly from the United States, but has also been found in Canada England and Australia Baker (1957) summarized the literature between 1943 and 1957 Since Baker s publication many other cases have been reported The disease as observed since 1943 has the following features It takes mainly cerebral and also pulmonary forms and rarely, intestinal ocular, and disseminated forms The cerebral disease is usually associated with orbital and paranasal sinus infection It occurs at all ages It is acute rather than

chronic and is usually fatal, although recovery in a few patients has been observed The disease usually is found as a complication of other diseases, of which uncontrolled diabetes is the commonest, but other diseases such as leukemia multiple myeloma, Hodgkin's disease, fatal burns, uremia, cirrhosis, and diarrhea are also antecedent conditions Cortisone, corticotropin, anti leukemic chemical agents, and antibiotics may also be predisposing factors (Baker, 1957) Growth of the fungus in the tissue is abundant Its spread is unique in that it has a great tendency to invade massively the tough arterial and venous walls, causing thrombosis that results in widespread necrosis of the tissue Cellular infiltration and abscess formation are seen This cellular reaction consists chiefly of neutrophils and macrophages It is believed that this cellular reaction is provoked by tissue necrosis, since hyphae are often found within the tissue without causing cellular infiltration Eosinophils were not reported to be a part of the tissue reaction, while multinucleated giant cells were rare and were reported in only one case (Baker, 1956) The fungus was isolated from three cases, and was found to belong to the genus *Rhizopus* (Harris, 1955 Bauer *et al*, 1955, Baker, 1957)

Phycomycosis affecting organs other than the subcutaneous tissue has been found twice in Indonesia One case presented an infection of the stomach wall (Lie Kian Joe and Njo Injo Tjoei Eng 1956) The fungus that was not cultured did not grow into the blood vessels The patient recovered after partial gastrectomy The second case presented a fatal infection of the central nervous system associated with orbital infection and uncontrolled diabetes mellitus (Lie Kian Joe *et al*, 1960) The fungus, which was not isolated grew abundantly and did invade blood vessels, but not massively A few eosinophils were seen in granulomatous tissue which, in association with abscesses, consisted of epithelioid histiocytes and multinucleated giant cells

Subcutaneous phycomycosis is unique in that it differs in many respects from phycomycosis affecting the other organs These differences are

(1) Subcutaneous phycomycosis has been found only in children There is probably an age disposition, although we can not be sure until more cases are available for study

(2) There are no known predisposing factors The disease occurs in healthy children, and there has been no association of it with the use of antibiotics or other drugs

(3) The disease is chronic, lasting for many months or many years without markedly affecting the general condition of the patient

(4) The infection heals spontaneously

(5) The disease produces an eosinophilic granulomatous tissue reaction

(6) The fungus causes a peculiar smudgy, eosinophilic, granular necrosis of the surrounding tissue not found in other mycotic infections

(7) The fungus has no tendency to grow into the blood vessels

(8) The fungus grows slowly in tissue, and usually hyphae occur singly

(9) Three strains of a *Basidiobolus* species and an unidentified phycomycete have been isolated

We do not know why spontaneous healing occurred in one case more than 4 years after the disease started and, in another, after 9 months This is a matter of balance between the defending host and the invading parasite, about which

we know nothing However, we can assume that spontaneous healing may also occur a short time after infection when the lesion is still small It may well be that such cases occur without recognition their diagnosis is extremely difficult, since they do not show symptoms

Subcutaneous phycomycosis has not been found in other countries The patient observed by W St C Symmers in London, England, had probably acquired the infection in Indonesia It is possible that the disease is not restricted to Indonesia alone, since the fungus is found in many areas of the world

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GROWTH CHARACTERISTICS OF THE FUNGI OF CHROMOBLASTOMYCOSIS*

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It is well known that five species of imperfect, dematiaceous fungi are now recognized as causing chromoblastomycosis These are *Phialophora verrucosa*, *Cladosporium carrionii* and three species of controversial generic position that some of us prefer to place in the separate genus, *Fonsecaea*[16], namely *Fungi pedrosoi*, *F compacta* and *F dermatitidis* These five species sporulate by three distinct methods of conidial production the *Cladosporium*, the pseudo *Acrotheca*, and the *Phialophora* types Since the three types of sporulation can occur simultaneously in various combinations and proportions, depending on the individual isolate and conditions of culture, it frequently takes a specialist to identify these pathogens

This paper does not repeat the criteria on which final identification is based nor the arguments in favor of one or another of the proposed taxonomic treatments for this group, rather, it attempts to collate the criteria, some old, some new, that the nonspecialist may find helpful in making an initial differentiation between the fungi of chromoblastomycosis and related saprophytes and pathogens This initial differentiation is necessary For one thing, various species of *Cladosporium* are frequently introduced as air contaminants into cultures of clinical specimens Such contaminants can no longer be discarded if they lack *Phialophora* or pseudo *Acrotheca* types of sporulation the discovery of an undisputed *Cladosporium* (*C carrionii*) as a frequent and widespread agent of chromoblastomycosis calls for other means of identification Another need for differentiation springs from the confusing resemblance of *P jeanselmei*, one of the causes of mycetoma, to the colony and microscopic morphology of the fungi of chromoblastomycosis The resemblance has led to erroneous identification of this fungus and confusion in clinical diagnosis Distinction between these two diseases is important if only for guiding therapy the cutaneous localization of chromoblastomycosis makes it amenable to plastic surgery, in maduromycosis on the contrary, plastic surgery is inapplicable since there the fungus invades subcutaneous tissue, muscle, cartilage, and bone Finally, it would also be helpful if a similar means of preliminary distinction could be found between *P verrucosa* and the saprophytic phialophoras that attack wood Although not yet reported as contaminants, conceivably such could happen

Evaluation of Previously Proposed Diagnostic Criteria

Sporulating pattern Since the fungi of chromoblastomycosis are yet to be rescued from the category of Fungi imperfecti, their asexual sporulation, as in all these form genera, is still the only legitimate basis for their identification As mentioned earlier, however, the correct evaluation of their complicated

* The work described in this paper was supported in part by Research Grant No E 299 (C7) from the Institute of Allergy and Infectious Diseases Public Health Service Bethesda Md

we know nothing However, we can assume that spontaneous healing may also occur a short time after infection when the lesion is still small It may well be that such cases occur without recognition their diagnosis is extremely difficult, since they do not show symptoms

Subcutaneous phycomycosis has not been found in other countries The patient observed by W St C Symmers in London, England, had probably acquired the infection in Indonesia It is possible that the disease is not restricted to Indonesia alone, since the fungus is found in many areas of the world

Acknowledgment

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GROWTH CHARACTERISTICS OF THE FUNGI OF CHROMOBLASTOMYCOSIS*

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It is well-known that five species of imperfect, dematiaceous fungi are now recognized as causing chromoblastomycosis These are *Phialophora verrucosa*, *Cladosporium carrionii* and three species of controversial generic position that some of us prefer to place in the separate genus, *Fonsecaea*[16], namely *Fungi pedrosoi*, *F compacta*, and *F dermatitidis* These five species sporulate by three distinct methods of conidial production the *Cladosporium*, the pseudo-*Acrotheca*, and the *Phialophora* types Since the three types of sporulation can occur simultaneously in various combinations and proportions, depending on the individual isolate and conditions of culture, it frequently takes a specialist to identify these pathogens

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pattern of conidial production requires experience and time-consuming observation For further details on this criterion, Carrión,[4,6] Emmons,[2] Conant,[10] and Trejos[23] may be consulted

Serological reactions A review of the serological reactions employed before 1947 to distinguish these fungi has been published[6,7] The most pertinent of these were the observations of Martin *et al*[14] and of Conant and Martin,[9] who showed serological cross reactivity among the fungi of chromoblastomycosis but not between them and *C sphaerospermum (langeronii)* Later, in 1958, Seeliger[18] reported that he and Trejos had found *P jeanselmei* antisera inactive against alcohol precipitated culture filtrates of the fungi of chromoblastomycosis Although serological criteria can be very useful taxonomically, here again a specialized laboratory is essential

Hyphal fusions The occurrence of hyphal fusions among the fungi of chromoblastomycosis was first reported by Trejos[23] in *C carrionii* Although Ridley[17] failed in her attempt to repeat this observation, Taschdjian[22] found intraspecific but not interspecific fusions when she tested 2 isolates each of *C carrionii*, *F pedrosoi*, *F compacta*, *F dermatitidis*, and *P verrucosa* This observation suggests another possible means of distinguishing between these species, but unless confirmed cytologically or by analysis of progeny, this method is not universally accepted as a criterion for the identification of species

Enzymatic reactions Work with the enzymatic reactions of the fungi of chromoblastomycosis was reviewed in 1947 by Carrión and Silva,[7] who concluded that previous tests were "too few and conflicting to permit conclusions" It was not until Montemayor's study of 1949[5] that enzymatic reactions were proposed as a means of differentiating between the pathogens and related saprophytes Proteolytic enzymes, that is, those liquefying Loeffler's coagulated serum medium or gelatin, and those peptonizing milk, were absent in the pathogens but present and very active in two saprophytic cladosporia used as controls

This criterion has also been used by Trejos[23] in describing *C carrionii* as a new species, by Ridley[17] in screening for pathogenic cladosporia among fourteen thermophilic isolates from soil and vegetable substrata in Australia

Optimal temperature Montemayor[15] had reported an optimal temperature of 20 to 25° C for 2 saprophytic cladosporia, of 29 to 30° C for 3 isolates of *P jeanselmei*, and of 37° C for 6 isolates from chromoblastomycosis Silva,[20] on the other hand, found the optimum temperature in the latter group to range between 25 and 35° C Further observations were indicated

Experimental inoculations Since the fungi of chromoblastomycosis are of very low infectivity for laboratory animals,[7,19] animal inoculation has not been a practical criterion for pathogenicity However, effective inoculation of three human volunteers was convincingly reported by Ridley[17] as one of the criteria for identifying as *C carrionii*, two isolates she had obtained from fence posts in Australia Although useful in Ridley's experience and that of certain other investigators, inoculation of humans is not the most popular or the safest method of identifying fungi

Search for Simpler Criteria

The above mentioned criteria, useful as they are in the hands of the specialist, are not only time consuming but require materials not generally available in laboratories devoted to isolation of fungi from clinical specimens Therefore, there is still need for additional criteria that could be employed at least in the primary differentiation between the fungi of chromoblastomycosis and related saprophytes or pathogens

My experience with these fungi has suggested that the rate of growth at 25° C, the ability to grow at or above 30° C, tolerance to cycloheximide, and growth stimulation by vitamins and dimorphism might be useful criteria for this preliminary differentiation

Materials and Methods

To verify this impression a study was conducted with 83 isolates from the combined stock collections of Carrión, Benham, and myself Of these, 64 were from chromoblastomycosis and included 47 of *Fonsecaea pedrosoi*, 4 of *F compacta* (2 clinical isolates and their variants), 1 of *F dermatitidis*, 5 of *C carrionii*, 5 of *P verrucosa* and 2 of "*Torula poikilospora*" (Takahashi) Eleven were related pathogens from other diseases and included 6 of *P jeanselmei* and 2 of *C gougerotii* [8] The remaining isolates were saprophytes or plant parasites and included 6 *Cladosporium* sp, *and* 1 each of *P richardsiae*, *P obscura Pullularia pullulans*, *C elatum* and *C sphaerospermum* (*langeronii*) (see TABLE 1)

The techniques employed in this study were the conventional ones pin point inoculations were made in the center of cotton plugged agar slants and growth was measured as colony diameter after three weeks incubation on the various media and at the temperatures studied

Growth rates were measured on 3 routine isolation media Sabouraud's glucose agar obtained in dehydrated form* but with the agar concentration raised by us to 2 per cent, a similar medium designated as "home made" Sabouraud's in which Bactopeptone was substituted for the Neopeptone of the commercial preparation (both these media contained 4 per cent glucose, 1 per cent peptone, and 2 per cent agar, final pH of 5.5), and a third medium containing cycloheximide (B B L's Mycosel Phytone, 1 per cent, glucose, 1 per cent, agar, 1 5 per cent, cycloheximide, 0 4 per cent, chloramphenicol, 0 05 per cent)

To determine the dimorphic morphology, Cystine Heart Hemoglobin† (CHH) agar slants in screw cap tubes were inoculated by smearing the entire surface of the medium with inoculum contained in a wire loop or sterile cotton swab Inocula were obtained from either a Sabouraud slant or a previous transfer on the cystine medium Three successive transfers (T_1, T_2, and T_3) were made at intervals of 1 week on the cystine media After 1 week's and again after 3 weeks' incubation of each of these subcultures at 37° C, their

* From Difco Laboratories Inc Detroit Mich
† Difco

microscopic morphology was examined by means of teased mounts and the degree of conversion to the parasitic growth phase recorded

To determine the effect on growth of inorganic versus organic nitrogen substrates, agar slants of a mineral-base medium were prepared according to

TABLE 1
ORIGIN OF CERTAIN ISOLATES STUDIED*

(1) Pathogens resembling the fungi of chromoblastomycosis
  (a) *Phialophora jeanselmei*
    563 Isolate obtained from the *Centraalbureau voor Schimmelcultures* (C B S) Baarn, Holland, in 1949, labeled as *Phialophora jeanselmei* (Langeron) Emmons L P, Negroni
    565 1 Isolate recovered in 1943 from case of mycetoma of the hand seen by G M Lewis and M E Hopper (their No 43 505)
    565 2 Isolate from second case of mycetoma seen in 1949 by Lewis and Hopper (their No 49-491)
    579 Isolate received in 1946 from C W Emmons (his No 8724)
    580 Isolate received in 1946 from Emmons (his No 8727)
    625 Isolate recovered in 1941 from same patient as isolate 565 1, both described by Lewis and Hopper as *F pedrosoi typicus*[16]
  (b) *Cladosporium gougerotii*
    590 Isolate from case seen by L S Suter, Kennedy Hospital Memphis Tenn (his No 1792), and described by Young and Ulrich as *Sporotrichum gougerotii*[27]
    1012 Isolate received from R Sabouraud s collection in 1929 labeled as *Sporotrichum gougerotii* Mlle Zelande
(2) Saprophytes resembling the fungi of chromoblastomycosis
  (a) Phialophoras
    656 *Phialophora obscura* isolated from wood pulp in Sweden and received from L W White (United States Quartermaster Culture Collection No QM 268)
    657 *Phialophora richardsiae* isolated from wood pulp in Sweden and received from the same source as above (QM 263)
  (b) Cladosporia
    593 *Cladosporium sphaerospermum (Hormodendrum langeroni)* (No 377 in A L Carrión's collection isolate included in studies by Emmons 1936)[28]
    600 *Cladosporium (Hormodendrum) elatum* (No 385 in Carrión s collection, isolate included in studies by Emmons, 1936)[28]
    603 *Cladosporium* sp Isolate believed to be a contaminant received from C Calero (Panama Canal Zone) in 1945
    604 *Cladosporium* sp isolated from leaves, 1935, labeled as saprophyte in Carrión's collection (his No 389)
    650 *Cladosporium* sp Isolate received from W H Weston in 1944 and labeled as Bouganville, B C 22 (a)
    651 *Cladosporium* sp Isolate received from Weston in 1944 and labeled Mold from carbathiazole
    697 *Cladosporium* sp Isolate received from E L Hazen in 1959, as a "saprophytic *Cladosporium*" (No 4893 in the collection from the New York State Health Department, Division of Laboratories and Research Albany, N Y)
    698 *Cladosporium* sp Isolate received in 1959 from L Ajello, believed to be a contaminant (C D C No My-374 59)

* List includes only those isolates from genera or species that failed to show uniform behavior (see *Results* and *Discussion*)

Georg and Camp,[12] to contain 4 per cent glucose, 0 01 per cent $MgSO_4 \cdot 7H_2O$, 0 18 per cent KH_2PO_4 (anhydrous), and 2 per cent agar* (not purified). Four modifications of this medium were prepared by adding (1) 0 15 per cent ammonium nitrate, (2) 0 15 per cent ammonium nitrate plus vitamin supplement, (3) 0 25 per cent casein hydrolysate, and (4) 0 25 per cent casein plus vitamin

supplement The casein hydrolysate was obtained as 10 per cent sterile solution,* the vitamin supplement was that previously employed[21] according to Arêa Leao and Cury[1] thiamine HCl, riboflavin, pyridoxine HCl, nicotinic acid, Ca pantothenate, 500 μg /l of each, inositol, 10,000 μg /l , biotin, 5 μg /l Incubation was at 25° C

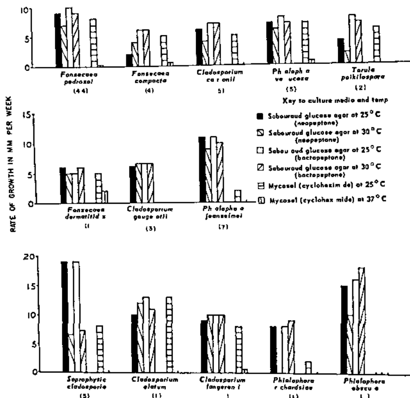


FIGURE 1 Rates of growth of the fungi of chromoblastomycosis and of related saprophytes and pathogens Total number of isolates examined of each species is shown in parenthesis rates shown represent arithmetic average

Results

Growth on routine isolation media FIGURES 1 and 2 summarize the rates of growth obtained on Sabouraud's glucose agar (with Bactopeptone versus Neopeptone) calculated from the colony diameters after 3 weeks' incubation at 25 and at 30° C The same illustrations also show the growth rates on the medium containing cycloheximide (Mycosel†), after three week's incubation at

* From Nutritional Biochemicals Co , Cleveland Ohio
† Baltimore Biological Laboratories, Baltimore, Md

25 and at 37° C All the isolates listed under *Materials and Methods* wer
employed in these measurements, except that on the cycloheximide medi
at 37° C, only 18 isolates were employed 2 each of *F pedrosoi*, *F compacta*
P verrucosa *C carrionii* and *C gougerotii*, 1 each of *F dermatitidis*, *P obscura*

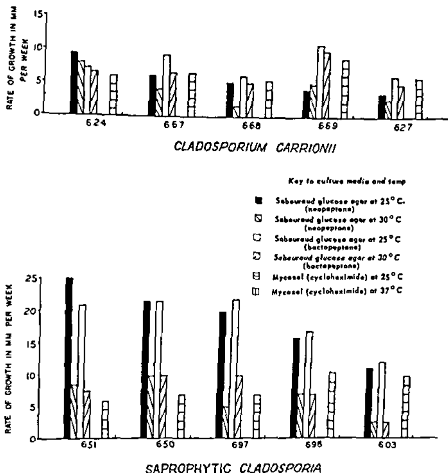


FIGURE 2 Comparison of rates of growth of five isolates each of *Cladosporium carrionii* and saprophytic cladosporia

P richardsiae, *C sphaerospermum* (*langeronii*), *C elatum*, *P pullulans*, and 2 of *P jeanselmei*

It will be seen that practically all isolates grew better at 25° than at 30° C, the exceptions being *C elatum*, *C sphaerospermum* (*langeronii*) and isolate No 669 of *C carrionii* (from E H Derrick, Brisbane, Australia) The more rapid growth rate at 25° C than at 30° C was particularly noticeable in the 5 saprophytic cladosporia shown in FIGURE 2 and in *P obscura* (FIGURE 1)

Another notable observation was the superiority of Bactopeptone over

Neopeptone as a substrate for growth Although the bar diagrams show this difference in growth rates calculated at the end of a three week period the difference was even more marked at the end of the first week of growth (FIGURE 3) Bactopeptone promoted a more rapid initiation of growth as well as larger colonies than Neopeptone

Growth on cycloheximide media at 25° C was remarkably good even for saprophytes here again there were some exceptions namely *P jeanselmei* *P obscura* and *P richardsiae* Growth was absent on this medium in practically

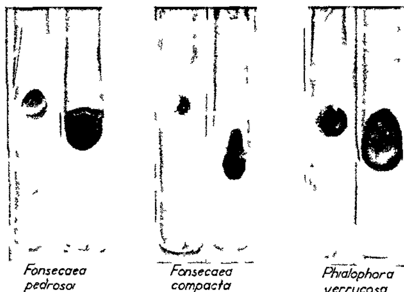


FIGURE 3 Sister colonies of three of the fungi of chromoblastomycosis after one week of growth at 25° C on Sabouraud s glucose agar made with Neopeptone (*left*) and with Bactopeptone (*right*)

all those cultures incubated at 37° C (exception *F dermatitidis*), but this can not be attributed to greater toxicity of cycloheximide at this temperature Another test read too late to be included in the bar diagrams revealed that none of the isolates that failed to grow on cycloheximide media at 37° C was able to grow at this temperature during a three week period on the home made Sabouraud s (Bactopeptone) *F dermatitidis* however grew on both these media although slowly attaining a diameter of 6 mm in 3 weeks

Growth and morphology on Cystine Heart Hemoglobin agar at 37° C On this medium 85 isolates were studied by the method described above Growth here was graded as follows ++++ represented confluent growth covering the entire surface of the agar +++ confluent growth limited to an area ranging from one half to three fourths the agar surface ++ several discreet colonies of at least 2 mm in diameter, + few colonies of only about 1 mm

in diameter, ±, colonies hardly larger than particles of inoculum That some growth had occurred even in the last category was verified by microscopic examination Of the 85 isolates, the growth of 41 was ++++, 10, +++, 8, ++, 18, +, and 8, ± Among the isolates that were able to grow on this medium at 37° C were included the 17 that had *failed* to grow on Sabouraud's Bactopeptone agar at this same temperature

The degree of conversion to the parasitic phase was disappointing, even though a progressive change took place from a glossy-velvety growth to a dull mealy surface texture with variable numbers of chlamydospores In no case,

Table 2
Response of Various Isolates to B Complex Vitamins and/or Organic Nitrogen*

Isolate No	Classification	Stimulation by B-complex vitamins	Stimulation by organic nitrogen
597, 607	*Fonsecaea pedrosoi*	+	0
553, 589	*Fonsecaea compacta*	+	0
614	*Fonsecaea dermatitidis*	+	0
624, 669	*Cladosporium carrionii*	+	0
590	*Cladosporium gougerotii*	+	0
593	*Cladosporium sphaerospermum*	+	0
600	*Cladosporium elatum*	+	0
653	*Phialophora jeanselmei*	+	0
657	*Phialophora richardsiae*	++	0
565 1 625	*Phialophora jeanselmei*	0	++
556, 639	*Phialophora verrucosa*	+	+
656	*Phialophora obscura*	++	+
671	*Pullularia pullulans*	0	0
563 579, 580	*Phialophora jeanselmei*	0	0
1012	*Cladosporium gougerotii*	0	0
603, 650, 697, 698, 651	Saprophytic cladosporia	0	0

Key ++ = supplement (or one of its components) *essential* for growth, + = supplement (or one of its components) beneficial to growth, 0 = supplement has no effect on growth
* Composition of media detailed in text

however, was conversion to the parasitic (sclerotic cell) phase as complete as had been previously observed on 2 isolates (one of *P verrucosa*, one of *F pedrosoi* included above) similarly cultured but on the original Francis cystine glucose blood agar[19] The differences between these 2 media and their possible effect on the induction of sclerotic cells need further study

Another interesting response was that shown by the so called black yeasts Although moldy on Sabouraud's glucose agar at room temperature, two of the isolates of *P jeanselmei* and one of *C gourgerotii* became glistening and yeast-like in gross and microscopic morphology when subcultured on the CHH agar at 37° C

Growth on inorganic versus organic nitrogen with and without B-complex vitamins The same selection of isolates tested for ability to grow on cycloheximide and Bactopeptone agars at 37° C, and 4 additional strains of *P*

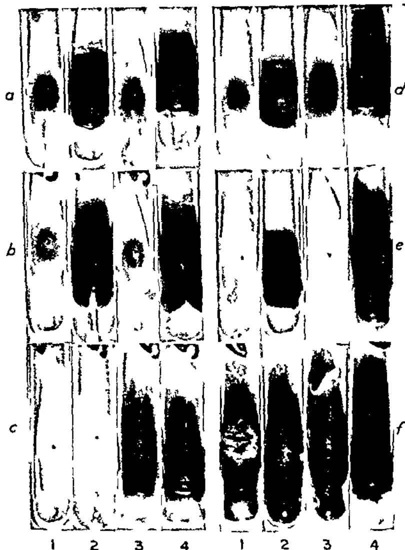


FIGURE 4 Response of six representative isolates to inorganic versus organic nitrogen substrate with and without vitamin supplement Columns 1 and 2 show tubes containing NH_4NO_3 as sole nitrogen source with added vitamins on the right Columns 3 and 4 show tubes containing casein as nitrogen source those with added vitamin on the right (a) *Fonsecaea pedrosoi* (b) *Cladosporium sphaerospermum (langeroni)* (c) *Phialophora jeanselmei* (d) *P verrucosa* (f) *P obscura* (g) *Pullularia pullulans*

jeanselmei were used in this preliminary survey of nutritional requirements on the 4 media described above As shown in TABLE 2, 4 types of response were obtained (1) stimulation by vitamins but not by organic nitrogen, (2) stimulation by organic nitrogen but not by vitamins (3) stimulation by both the vitamin supplement *and* organic nitrogen, and (4) no stimulation by either of the 2 enrichments Type 1 response was shown by each of 2 isolates of *Fon secaea pedrosoi* (FIGURE 4*a*), *F compacta C carrionii*, and by single isolates of *C gougerotii*, *F dermatitidis*, *P jeanselmei*, *P richardsiae*, *C sphaerospermum (langeronii)* (FIGURE 4*b*), *C elatum* and a saprophytic *Cladosporium* from leaves The 2 saprophytic phialophoras, *P obscura* and *P richardsiae*, have an absolute deficiency for an undetermined component of the vitamin supplement Type 2 response was demonstrated with 2 of 6 strains of *P jeanselmei* (both from the same patient, 1 strain illustrated in FIGURE 4*c*) Here too the requirement for organic nitrogen was absolute Type 3 response was shown by the 2 isolates of *P verrucosa* (FIGURE 4*d*) and by one of *P obscura* (FIGURE 4*e*) Type 4 response, that is equal growth on all 4 media, was shown by 5 saprophytic cladosporia, by *Pullularia pullulans* (FIGURE 4*f*), by the remaining 3 isolates of *P jeanselmei*, and by the remaining isolate of *C gougerotii*

Discussion

Some of the results of the studies described herein are not entirely in accord with commonly accepted ideas For example, the general impression that the faster growth rate at 25° C of the air borne contaminants of *Cladosporium* than that of the fungi of chromoblastomycosis provided a reliable criterion for differentiation of these 2 groups was not borne out in our experiments In our study, 4 saprophytic isolates of *Cladosporium* (Nos 650, 651, 697, and 698) grew very rapidly, at rates ranging from 16 to 25 mm /week, attaining colony diameters ranging from 50 to 75 mm after 3 weeks on Sabouraud s glucose agar at 25° C, while 4 others Nos 593, 600 603, and 604 grew more slowly, at a rate fully comparable to the rates shown by the fungi of chromoblastomycosis (9 to 11 mm /week) The conclusion, then, is that while a fast growing *Cladosporium* probably spells saprophyte, a slow growing one is not necessarily pathogenic for man Indeed, the wide range of variation of growth rate with the saprophytes in this genus had already become evident from comparative studies such as those described by DeVries[24]

The second criterion examined, whether saprophytes would cease to grow or would slow down considerably in rate of growth at 30° C, gave a similar answer Absence of growth or extreme slowing at 30° C was found to be a fairly good sign of a saprophyte Ability to grow well at this temperature however, was not found to be the exclusive prerogative of a pathogen

Many years ago I observed the superiority of Bactopeptone over Neopeptone as an ingredient in Sabouraud's glucose agar intended for cultivation of the fungi of chromoblastomycosis, this observation has been confirmed on a large number of isolates Since this isolation and maintenance medium is widely marketed with Neopeptone, it would seem advisable to substitute a "homemade" medium prepared with the more favorable Bactopeptone when the medium is intended for use with the fungi of chromoblastomycosis In ad

dition to a more rapid and luxuriant growth, Bactopeptone prolongs the viability of cultures of this group maintained in a stock culture collection

Growth of the fungi of chromoblastomycosis on cycloheximide medium was a surprise, since their low virulence and ability to grow well on inorganic nitrogen has often been construed as a sign of weak pathogenicity That this group could tolerate, and even "be stimulated by" cycloheximide was pointed out to me by Lucille Georg Tolerance but not stimulation by this antibiotic has been confirmed with almost all the 85 isolates studied Because this antibiotic is known to inhibit saprophytic fungi,[11 25] it had been hoped that this compound might serve in ruling out of saprophytes frequently confused with the fungi of chromoblastomycosis With the exception of one isolate of *P obscura*, all other saprophytic isolates tested grew well on this antibiotic Cycloheximide, therefore is not suitable for the proposed preliminary separation of saprophyte from pathogen in this instance

The good growth shown on CHH agar at 37° C by more than 60 isolates, including 17 that had failed to grow at this temperature even in the favorable Bactopeptone Sabouraud's agar was provocative The possibility should be explored that temperature tolerance in these fungi may also be affected by growth factors such as those identified for various protists by Hutner *et al*[13]

The observation of a marked stimulation of the fungi of chromoblastomycosis by the vitamin supplement confirms that of Arêa Leao and Cury[1] who identified thiamin as the responsible factor

Finally, marked variation was observed among isolates sent to us as *P jeanselmei* or *C gougerotii* both in their response to CHH agar (mealy with chlamydospores versus yeasty with blastospores), and in their response to organic nitrogen and to B complex vitamins (stimulated or not stimulated) This observation opens up the possibility that more than one species may be represented among isolates now classified as either *P jeanselmei* or *C gougerotii* The recommendation of Borelli[3] that these two species be considered synonymous should await further study

Summary

This paper reviews and proposes criteria that may serve in the preliminary differentiation between the fungi of chromoblastomycosis and related saprophytes and pathogens In the study 83 isolates were examined among which *Fonsecaea pedrosoi*, *F compacta*, *F dermatitidis*, *C carrionii*, *P verrucosa*, and *Torula poikilospora*," served as representatives of etiologic agents of chromoblastomycosis, *P jeanselmei* and *C gougerotii* of causative agents of mycetoma *C sphaerospermum* (*langeronii*), *C elatum*, *P obscura*, *P richardsiae*, *Pullularia pullulans* and six other isolates assigned to *Cladosporium*, represented the saprophytes

Confirmation was obtained that whereas some air contaminants of the genus *Cladosporium* may grow at a rate considerably faster than the fungi of chromoblastomycosis, other saprophytic cladosporia grow at rates comparable to known pathogens

Bactopeptone was shown to be superior to Neopeptone as an ingredient for

Sabouraud's glucose agar intended for use with the fungi of chromoblastomycosis

With few exceptions, saprophytes as well as pathogens in this study grew well on cycloheximide at 25° C

Although 17 isolates representing a cross section of the various species studied failed to grow at 37° C on Sabouraud's Bactopeptone agar, these same isolates and nearly 50 others attained moderate to luxuriant growth at this same temperature on CHH agar

Serial streaking of mold phase cultures of 85 isolates on CHH agar incubated at 37° C induced partial conversion to the parasitic morphology This was manifested by a gradual change from a smooth velvety to a dull mealy type of aerial mycelium and the appearance of numerous, sometimes septate, chlamydospores along the mycelium Complete suppression of filaments was not attained, except with some isolates of *P jeanselmei* and one of *C gougerotii* where conversion was from a velvety filementous to a smooth yeastlike type of aerial growth consisting entirely of blastospores

Two isolates each of four species causing chromoblastomycosis and one each of *C gougerotii*, *C elatum* *C sphaerospermum (langeronii)*, *P jeanselmei*, and *P richardsiae* grew equally well on inorganic and organic nitrogen but were markedly stimulated when a supplement of B complex vitamins was added to the medium Two isolates of *P verrucosa* and one of *P obscura* were stimulated by both organic nitrogen and the vitamin supplement Two isolates of *P jeanselmei* (from the same patient but different laboratories) did not grow on inorganic nitrogen even when vitamins were added, but grew as well on organic nitrogen (casein) with or without vitamins Three other isolates classified elsewhere as *P jeanselmei* one of *C gougerotii* and one of *P pullulans* grew as well on inorganic as organic nitrogen with or without vitamins

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GEOGRAPHIC DISTRIBUTION AND PREVALENCE OF THE DERMATOPHYTES

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A survey of published reports on twenty well defined dermatophyte species reveals that, while some species are cosmopolitan, others have a remarkably limited geographic distribution (TABLE 1)

The dermatophytes can be classified into three broad categories the anthropophilic, the zoophilic, and the geophilic species [1] This differentiation with respect to natural habitats and host preferences is believed to have played a significant role in determining the global distributions of the dermatophytes

Of the eleven species that are cosmopolitan, seven are anthropophilic *Epidermophyton floccosum Microsporum audouinii*, *Trichophyton mentagrophytes* (downy form), *T rubrum*, *T schoenleinii*, *T tonsurans*, and *T violaceum* Because of their adaptability to a wide range of environmental conditions and their intimate association with man, these fungi occur wherever man dwells

The animals that man domesticated also became hosts for certain dermatophytic fungi As man and his domestic animals migrated throughout the world, some of the zoophilic species, *M canis*, *T gallinae*, *T mentagrophytes* (granular form) and *T verrucosum*, accompanied them

M gypseum is the only geophilic dermatophyte with a global distribution The factors that established its geographic pattern are not known

Six anthropophilic dermatophytes have a limited geographic distribution *M nanum*, *T concentricum*, *T ferrugineum*, *T megnini*, *T soudanense* and *T yaoundei* Four of these species are confined to single regions of the world At present, *M nanum* is known from only two Cuban isolates [2] *T megnini*[3, 4] is confined exclusively to Europe It has been recorded in Denmark,[5] France,[6] Germany,[7] Great Britain,[8] Hungary,[9] Italy,[10] the Netherlands,[11] Portugal,[12] Union of Soviet Socialist Republics,[13] and Spain[14] It is especially common on the island of Sardinia [10]

Africa has two endemic species *T soudanense* and *T yaoundei* *T soudanense* occurs in Algeria,[15] Angola (L K Georg, personal communication) Cameroon,[16] French West Africa,[17] and Nigeria,[18] but there is also a record of the recovery of *T soudanesne* from a child in Brazil (L K Georg, personal communication) Its presence there may be a legacy of the infamous slave trade that flourished from the Fifteenth to the Nineteenth Centuries This endothrix fungus superficially resembles *T tonsurans* and, until it is deliberately sought in Brazil, its true status will remain unknown

T yaoundei was first described in 1957 from the French Cameroons,[16] now the Republic of Cameroon There it proved to be one of the principal agents of tinea capitis Subsequently, it has been found to be present in the Belgian Congo (L K Georg, personal communication)

T concentricum is recorded from Oceania, Asia, and North, Central, and South America [19] It is not present in the New World north of Mexico and it is absent from Europe and Africa A supposed isolate of this species from

Africa,[20] when studied by Georg, proved to be *T verrucosum*[21] Pijper's report of this fungus in South Africa is erroneous,[22] as the published descriptions of his isolate indicate that he was dealing with a saprophyte

T ferrugineum is present in Africa, in Angola,[23] Belgian Congo,[24] Cameroon,[16] Cape Verde Islands,[25] in Asia, in China,[26] Formosa,[27] Hawaii,[27] India,[28] Japan,[29] Samoa[27] Vietnam,[27] and in Europe, in Bulgaria,[30] Czechoslovakia,[31] Hungary,[30] Italy,[33] Poland,[32] Union of Soviet Socialist Republics,[34] and Yugoslavia[35] This fungus is especially common in the Far East and parts of Africa During World War II it apparently was introduced from Manchuria and Korea into several European countries In Hungary, Poland, and the Union of Soviet Socialist Republics, it has become established in the native population although there is evidence that endemic foci existed in Bulgaria and Yugoslavia prior to World War II (L Ozegovic, personal communication)

TABLE 1
GEOGRAPHIC CLASSIFICATION OF THE DERMATOPHYTES

Cosmopolitan species			Geographically limited species		
Anthropophilic	Zoophilic	Geophilic	Anthropophilic	Zoophilic	Geophilic
E floccosum *M audouinii* *T mentagrophytes** *T rubrum* *T schoenleinii* *T tonsurans* *T violaceum*	*M canis* *T gallinae* *T mentagrophytes** *T verrucosum*	*M gypseum*	*M nanum* *T concentricum* *T ferrugineum* *T megninii* *T soudanense* *T yaoundei*	*M distortum* *T equinum*	*K ajelloi*

* *T mentagrophytes* is equally prevalent as a parasite of man and lower animals The downy form is associated with chronic human infections The granular form is associated with animal ringworm and acute human infections

An isolate of *T ferrugenium* from Uruguay,[36] confirmed by Georg, leads one to suspect that small endemic foci of this fungus occur in South America If this proves to be true, its presence in the New World may be another relic of the slave trade

M distortum and *T equinum* are the two zoophilic dermatophytes with apparently restricted distribution *M distortum* is known to occur only in New Zealand and the United States[37 38] The fact that the American isolates were recovered from monkeys newly imported from Latin America, and from humans who were in contact with those monkeys, suggests that this fungus is present also in Central and South America

T equinum, essentially a parasite of horses and related animals, is known in Europe in Belgium France, Germany, Great Britain, the Netherlands,[40] Italy,[41] and Spain[42] in North America, in Canada[40] and the United States,[40] and in South America, in Argentina, Uruguay,[40] and Brazil[41] It has not been recorded from equines in Africa Asia, and Australasia *T equinum* probably exists in those continents but, until animal ringworm surveys are carried out, its status will remain unknown

Table 2

Dominant Etiological Agent of Tinea Capitis in Various Areas of the World

Area / Country	Dominant agent
North America	
Canada	*M audouinii*[61]
United States	*M audouinii*[62]
Mexico	*T tonsurans*[63]
Caribbean area	
Cuba	*M canis*[64]
Puerto Rico	*M canis*[65]
South America	
Argentina	*M canis*[66]
Brazil	*T violaceum*[67]
Chile	*M canis*[68]
Peru	*T tonsurans*[69]
Uruguay	*M canis*[70]
Venezuela	*T tonsurans*[71]
Europe	
Denmark	*M canis*[72]
Finland	*M canis*[73]
France	*M canis*[74]
Great Britain	*M canis*[75]
Greenland	*T schoenleinii*[76]
Italy	*T violaceum*[77]
Portugal	*T violaceum*[78]
U S S R	*T violaceum*[79]
Spain	*T violaceum*[80–81]
Yugoslavia	*T violaceum*[80]
Africa	
Algeria	*T violaceum*[82]
Angola	*T ferrugineum* (L Re personal communication)
Belgian Congo	*T ferrugineum*[83]
Cameroon	*T yaoundei*[16]
Cape Verde Islands	*M canis*[84]
Egypt	*T violaceum*[82]
Morocco	*T schoenleinii*[82]
Nigeria	*M audouinii*[18]
Spanish Morocco	*T schoenleinii*[82]
Tripoli	*T violaceum*[82]
Tunisia	*T violaceum*[82]
Asia	
China	*T violaceum*[85]
India	Tinea capitis rare[84]
Iran	*T schoenleinii*[86]
Israel	*T violaceum*[87]
Japan	*T ferrugineum*[88]
Turkey	*T schoenleinii*[89]
Australasia	
Australia	*M canis*[90]
New Zealand	*M canis*[91]

Keratinomyces ajelloi, a geophilic fungus recently found to be pathogenic, has been recovered from soil and animals in North America and Central America in Alaska,[43] Canada,[43] United States,[43] Costa Rica,[44] in Europe, in Belgium,[43] Czechoslovakia,[43] France,[43] Germany,[45] Great Britain,[47] Hungary,[46]

Rumania;[47] in Asia, in Japan,[48] and in Australasia, in Australia,[43] and New Zealand.[43] As yet it has not been encountered in South America and Africa. As soil studies are carried out in those two regions, this fungus will undoubtedly be found to exist there.

This summary of the geographic distribution of the dermatophytes should not be considered definitive in any sense of the word, for it is based on incomplete data. The dermatophyte flora of many more regions of the world must be studied adequately before the distribution of these fungi will be known fully. More surveys, such as that of Pinetti on the dermatophyte flora of Italy,[53] are needed to fill the gaps in our knowledge.

Information on the prevalence of dermatophytes is even more incomplete than that on geographic distribution. Since the dermatophytoses are not reportable diseases, published reports can provide only fragmentary information concerning total numbers of infections and their specific agents. There is evidence that the predominant species differ not only from region to region, but may change with the passage of time. One must also take care to distinguish between the prevalence of fungus infections and the prevalence of dermatophytes in the environment. *K. ajelloi*[13, 49] and *M. gypseum*[49, 50] are abundant in soil, as indicated by the high percentage of soil isolates from many parts of the world, yet the number of infections that they cause is extremely low.

Despite the absence of adequate statistics, there is no doubt that the dermatomycoses are among the most common of human diseases. Tinea pedis afflicts millions of individuals. The principal etiologic agents involved in this disease in all parts of the world are *T. mentagrophytes* and *T. rubrum*.

The etiological agents of the other types of dermatomycoses—tinea capitis, tinea corporis, and tinea unguium—vary from region to region. This is illustrated in the tabulation of the predominant agent of tinea capitis present in various countries of the world (TABLE 2).

The number of children and adults suffering from ringworm infections of the body, nails, and scalp must be numbered in the millions. In the United States alone it has been estimated that public expenditures for ringworm medications approximate $25,000,000 per year (L. Ajello, unpublished data). The severity of postwar epidemics of tineas caused Yugoslavia to organize a nationally directed campaign against ringworm diseases that involved the establishment of a Mycological Observation Center to carry out a massive screening and therapeutic program.[52, 53]

It is evident that dermatophytes are widely disseminated in the environment in all parts of the world and that human and animal infections are extremely numerous.

Discussion

This essay on the geographic distribution and prevalence of dermatophytes has been an attempt to accomplish an impossible task: to draw a picture of a dynamic situation. The patterns of geographic distribution and prevalence of dermatophytes are not fixed. They are constantly changing under the influences of various forces such as climate, man's social and antisocial activities, his cultural habits, his migrations, and developments in therapy.

Two world wars have produced changes that the present generation of medical mycologists are still observing. The prevalence of tinea pedis in-

creased tremendously in the male population of Europe and the United States as a direct consequence of wartime conditions. The communal life of the military and reduced opportunities for maintaining high personal standards of hygiene created conditions that lowered resistance to infection and increased the opportunity for transfer of infections. In addition the warm humid climatic conditions found in the Pacific Theatre during World War II exacerbated previously existing ringworm infections and favored the spread of infection.

Civilian populations were also affected as large scale epidemics of tinea capitis broke out in many countries of the world. This brought about a change in the relative prevalence of species. In the United States, *M. audouinii* replaced *M. canis* as the predominant agent of tinea capitis.[27] Reports from Europe and the United States document a similar change in the relationship between *T. mentagrophytes* and *T. rubrum*. *T. rubrum* infections have significantly increased in number since World War II, with the result that they outnumber those caused by *T. mentagrophytes*.[93,94]

The late war is also responsible for the development of endemic foci of *T. ferrugineum* in the western part of the Union of Soviet Socialist Republics, Poland, Hungary and Czechoslovakia. Soldiers returning from the Far Eastern Front and Korean orphans given asylum in Eastern Europe who were infected by this dermatophyte introduced *T. ferrugineum* and it became established in the native population.

In the United States, migrations of farm laborers from Mexico and immigrants from Puerto Rico introduced *T. tonsurans* into the country on such a massive scale that this dermatophyte has become established in the native population.

The vast slave trade of the Fifteenth to the Nineteenth Centuries that brought millions of Africans into the Americas undoubtedly introduced new dermatophyte species into the New World. However, at this late date it is impossible to cite specific examples with the possible exception of *T. ferrugineum* and *T. soudanense*.

The geographic distribution of *T. concentricum* may be of anthropological significance. This fungus is widespread among the inhabitants of Polynesia and the countries bordering the western shores of the Pacific Ocean. However, it is only a sporadic parasite of Indians living in the tropical forests of Brazil, Guatemala, Mexico and San Salvador. The disparity in prevalence between the Asian endemic areas and those of Latin America leads me to speculate that the fungus was introduced from Asia into the New World. If this supposition has validity it tends to contradict Thor Heyerdahl's thesis that Polynesia was populated by pre-Columbian South American voyagers.[95]

Social habits also influence the occurrence of dermatophyte infections. Shoe-wearing people are apt to develop tinea pedis to a greater extent than those who customarily walk barefooted. Other habits such as the use of vegetable oils as hair dressing may account for the rarity of tinea capitis in India in contrast to the situation that prevails in European countries, the United States and elsewhere.

As the dermatomycoses affected more and more of the world's population vigorous measures to stop the spread of infection and to institute therapy were

taken Significant decreases in the prevalence of infections have been brought about by X ray treatment of tinea capitis in Europe Recently the antibiotic griseofulvin has been shown to be effective in curing all types of ringworm infection [96 98] If its therapeutic promise is sustained, the geographic distribution of the dermatophytes and the prevalence of infections will be changed radically in a few years

Note Since this manuscript was submitted for publication J C Gentles University of Glasgow Department of Medical Mycology Anderson College Glasgow Scotland informed me that he had received several cultures of *M nanum* isolated from pigs in Kenya, Africa Their identification was verified by L K Georg of this laboratory

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THE EFFECTS OF CORTISONE ON EXPERIMENTAL FUNGUS INFECTIONS*

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The administration of adrenal glucocorticoids clearly enhances experimental infections due to a wide variety of microbial agents[1] Numerous studies have been performed in which fungi have been used as the challenge microorganism In most of these investigations enhancement of infection was observed[2,3]

Although these studies offered definite evidence that adrenal steroids can augment experimental mycotic infections, they did not provide information as to the relative propensity for various fungi to spread under the influence of cortisone and did not adduce convincing data on the mechanisms of steroid action

The experiments summarized below were designed to delineate differences among fungi in the extent of cortisone enhancement Groups of mice were infected intravenously with one of three fungi that had been recovered from infections in man

Histoplasma capsulatum strain 6624, was isolated from a patient with disseminated histoplasmosis in 1952 *Cryptococcus neoformans*, strain 1148 a large capsule strain was recovered from the cerebrospinal fluid of a patient with chronic meningitis in 1954 *Candida albicans*, strain 851, had been cultured from the oropharynx of a patient with mucocutaneous moniliasis in 1956 These 3 strains had not altered in animal pathogenicity during serial passage on Sabouraud s agar The inoculum size varied from 3×10^4 to 10^7 cells per mouse Cortisone was administered in dosages of 0 1 to 2 0 mg intraperitoneally or subcutaneously The experimental period varied from 28 to 35 days

When cortisone was administered intraperitoneally starting 6 days prior to or the day after infection with *H capsulatum* and was continued daily for 28 days after infection the over all mortality and the rapidity of death were similar in cortisone treated and control animals at each inoculum

The results with cryptococcus were similar No increase in over all mortality and no alteration in the rapidity of death occurred in cortisone treated mice infected with various inocula from 2×10^4 to $2\ 5 \times 10^6$ cells

The results were strikingly different in mice infected with *C albicans* A marked increase in mortality occurred in cortisone treated mice infected intravenously with 1×10^6 to 5×10^6 cells Thus when 0 5 to 2 0 mg of cortisone was administered daily, starting 2 to 6 days prior to infection with 5×10^6 cells the mortality was 10 per cent in normal mice and 90 per cent in cortisone treated animals When treatment was started the day after infection, the mortality was 70 per cent The administration of small amounts of cortisone produced no significant increase in mortality

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It seemed clear that, under similar experimental conditions, cortisone enhanced monilia infections to a far greater degree than infections due to *H capsulatum* or *C neoformans*

Studies were then designed to analyze the tissue site of cortisone augmentation of mouse moniliasis, and to analyze the mechanisms underlying such enhancement

Fungus census was determined in various tissues of control mice 5 min to 60 days after infection with 1×10^5 to 5×10^6 cells Progression of infection occurred only in the kidneys with these inocula, and clearance of fungi was slowest in this organ Thus, in 14 to 23 per cent of normal mice, renal census rose during the first 2 weeks of infection more than 2 standard deviations above the mean observed during the initial 24 hours of infection In most mice, *Candida* populations remained stationary or fell progressively

In the liver spleen heart, lung, and brain there was a progressive fall in fungus census, so that 2 weeks after infection all tissues were sterile

When 0 5 mg of cortisone was given, starting 2 days prior to or the day following infection and was continued until 7 days after infection there was a marked and uniform elevation of renal census The administration of 0 1 mg of cortisone was not associated with significant increase in kidney titers

This is shown in FIGURE 1, which depicts studies in which an inoculum of 10^5 cells was employed Renal populations were determined at the termination of steroid therapy

In contrast, there was no evidence of progression of infection in the liver, lung, spleen and brains The administration of cortisone delayed fungus clearance, but in no animal was there evidence of significant increase in fungus titers as compared to those found 24 hours after infection

Studies on the lung and liver are summarized in FIGURE 2

The results of population studies on the heart were slightly different In 5 per cent of animals, there was clear increase in *Candida* titers under the influence of cortisone In the other 95 per cent clearance was commonly delayed but monilia census did not increase as compared to those found during the first 24 hours of infections

Thus, in cortisone-treated mice, progression of infection was confined almost exclusively to the kidneys, the organ of maximum infection in normal mice Further tissue census studies were performed in cortisone treated moribund mice dying within the first 7 days of infection with 5×10^6 cells Increase in monilia titers was maximum in the kidneys but also occurred to a significant degree with regularity in the heart

The bacteriological investigations summarized above suggested that there must be something unique in the host parasite relationship within the kidneys to establish this organ as the only site of progressive infection in normal mice and as the site of maximum enhancement in cortisonized animals

Therefore, serial histological studies were made of many tissues from normal and steroid treated animals including heart lung, brain, kidney, liver, spleen, muscle, adrenals, and pancreas The findings appear to offer a plausible explanation for the renal localization of experimental moniliasis

Following intravenous injection of 10^5 to 5×10^6 cells in normal mice, fungus cells were trapped in the capillaries of all tissues studied During the

first 12 hours of infection the fungus multiplied within these vessels and then broke into the interstitial tissues, where growth continued. A considerable polymorphonuclear leukocyte response occurred rapidly, and fungi could be seen to be phagocytized by leukocytes within 30 min of inoculation. Morphologic events were similar in all tissues during the first few hours of infection.

Thereafter, histological events in the kidney were strikingly different from those in any other tissue studied. Twelve to 24 hours after infection, fungi in

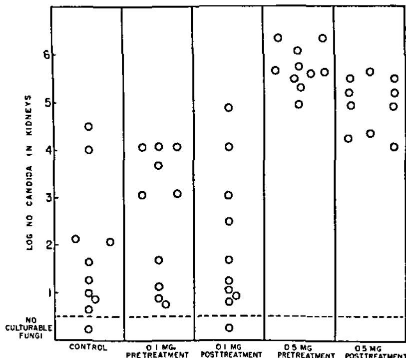


FIGURE 1 Effect of cortisone on kidney populations in mice infected I V with 10^6 cells of *Candida albicans*. Cortisone was administered subcutaneously 2 days prior to or on the day following infection and was continued daily until sacrifice 7 days after infection.

the interstitial tissues of the kidney ruptured into both cortical and medullary tubules. Intratubular residence appeared to protect the fungus from host cellular defenses. The *Candida* cells grew rapidly within the intratubular environment and formed long filaments. This growth was not associated with significant leukocyte infiltration into tubular lumens.

Only when the fungus ruptured back into the interstitial tissue were host defenses effective. Then massive polymorphonuclear leukocyte reaction and abscess formation occurred. The extent of intratubular growth appeared to determine the outcome of the infection. When it was limited, abscesses healed and the mouse recovered. When it was extensive, large coalescent ab-

scesses formed, with severe reduction in the amount of viable renal tissue and subsequent death of the animal In normal mice infected with up to 5 X 10^6 cells extensive intratubular rupture and growth were uncommon

FIGURE 2 Effect of cortisone on liver and lung populations in mice infected I V with 10^5 cells of *Candida albicans* Cortisone was administered in dosage of 0 5 mg per day beginning 2 days prior to infection and was continued until sacrifice 7 days after infection

In all other tissues studied there appeared to be no site for protected fungus growth Polymorphonuclear leukocytes appeared in large numbers and appeared to contain the infection Host defenses invariably prevailed with steady regression of lesions between 24 and 72 hours after inoculation

In cortisone treated mice candida growth was increased in interstitial areas of all tissues and this was associated with an increased inflammatory response

during the first 12 hours of infection The enhanced interstitial growth was accompanied by a marked increase in the extent of rupture of fungus cells into renal tubular lumens Subsequent abscess formation was likewise strikingly increased The greater degree of intratubular residence and consequent abscess formation appeared adequate to explain the increased kidney *Candida* census, as well as the increased mortality in steroid treated animals

The enhanced interstitial monilia growth and augmented cellular response in other tissues were associated with limitation and eventual eradication of the parasite, although this occurred somewhat slower than in normal animals

Thus, it would appear that the renal localization of moniliasis in normal mice and the limitation of enhanced infection almost exclusively to the kidneys in cortisone treated mice infected with strain 851 can be explained readily by the ability of the parasite to gain access to the renal tubular lumen and to grow within this protected site This hypothesis as to the pathogenesis and localization of candida infections is summarized in FIGURE 3

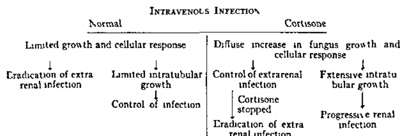


FIGURE 3 The pathogenesis of *Candida* infections in normal and cortisone treated mice

These studies were performed as indicated, with a single strain of *C albicans* This strain is considerably less virulent for mice than other strains such as the Fuentes strain used by Adriano and Schwarz[10] or those studied by Blyth,[11, 12] which produced significant mortality after the intravenous or intraperitoneal inoculation of 10^5 cells into normal animals The kidneys, according to these histological investigations, were the site of major involvement The renal lesions reported in Blyth's studies on normal mice were very similar to those described in the present experiments in mice treated with cortisone The histological abnormalities observed following inoculation of strain 851 into normal mice were also similar to those noted by Blyth, but were far less extensive

In mice dying within 2 to 4 days of infection with the more virulent strains, however, extensive lesions were also found in the heart and, in the case of the Fuentes strain, in the brain It is of interest that similar myocardial pathology was seen in our studies only in cortisone treated mice dying within 7 days of infection with 5×10^6 cells of strain 851

Thus, the administration of cortisone to animals infected with less virulent strain 851 enhanced the pathogenicity so that lesions were produced in the kidney, the organ of maximum infection, and occasionally, in the heart, that

were similar in nature and extent to those seen in normal mice infected with more virulent strains. The mechanisms of renal localization of progressive infection in normal or cortisone-treated animals infected with less virulent strain 851 or normal mice inoculated with the more virulent strains of Blyth appear to be similar and, in each case, are related to intratubular residence and growth.

The development of long filamentous forms was characteristic of intratubular growth. In contrast, early interstitial multiplication in kidneys and other tissues examined was predominantly in the yeast phase with small pseudomycelia. The problems of dimorphism in the growth of fungus pathogens *in vitro* and *in vivo* has been a subject of considerable debate. Young[13] and Blyth[11] have stated that the mycelial phase is necessary for the progression of *Candida* infection within tissues. On the other hand, many authors have argued that pathogenic fungi exist within tissues of warm blooded animals in the yeast phase, and that this phase is necessary for pathogenicity. The mycelial phase is supposedly controlled more readily by host defenses. This thesis and the phenomenon of dimorphism have been well summarized by Sherr and Weaver.[14] Sherr has also offered the hypothesis that fungus infections may possibly be controlled by alteration in the environment and metabolism of the parasite to compel its transformation into the less pathogenic mycelial phase.[15]

Our studies do not answer the question of the relative importance of the yeast and filamentous forms in the pathogenicity of dimorphic fungi. They do suggest that the persistence of *Candida* in tissues appears to be at least partly related to the formation of filaments within tubular lumens. It appears perfectly evident, on the other hand, that certain other fungi such as *H. capsulatum* or *C. immitis* are pathogenic almost invariably in the yeast phase. It seems likely, therefore, that both the mycelial and yeast can be of major importance in the persistence and progression of deep seated mycotic infections caused by fungi with dimorphic growth cycles. The relative importance of the two types of growth in individual mycotic infections would appear to be dependent on the environment created by the host for the parasite and on the nature and adaptability of the invading fungus. In experimental candidiasis due to strain 851, the host parasite interrelationship clearly favors the host in normal mice. The fungus can alter the host parasite relationship to favor persistence and, in some cases, progressive multiplication only by gaining access to the protected environment of the renal tubule and by forming long filaments in this site that subsequently form nonphagocytable foci around which abscesses develop.

In the experiments described above, we have investigated the relatively late events, occurring at least 12 to 24 hours after infection, that appear to be unique to the host parasite relationship in moniliasis and determine the localization of candida infections to the kidneys in normal and cortisonized mice. These investigations have not dealt thoroughly with the early generalized increase in interstitial *Candida* growth and cellular response during the initial 12 hours of infection under the influence of cortisone. This augmented interstitial fungus multiplication appears to determine the extent of subsequent intratubular rupture and growth. The mechanisms underlying such en

hancement are probably not unique to candidiasis, but are applicable to the enhancing effects of steroids on many infections

Numerous theories have been proposed to explain the adverse effects of cortisone on infection Each has some supporting experimental evidence These hypotheses include decreased mobilization of phagocytes,[16] diminished phagocytosis,[17] impaired killing within phagocytes,[18] depression of the inflammatory response [19] impaired reticuloendothelial function [20] diminished antibody production [21] decreased circulating antibody or opsonins,[22] abnormalities of antigen antibody interaction,[23] and decreased vascular permeability [24] It is also possible that potassium depletion or altered carbohydrate metabolism may be of importance

Preliminary quantitative studies of mouse myositis and peritonitis suggest that delayed mobilization of inflammatory cells may be of major significance When 5×10^7 cells of *C albicans* are injected intraperitoneally, the numbers of inflammatory cells appearing in the peritoneal fluid in cortisonized animals during the first 3 hours of infection vary from 15 to 40 per cent of those found in normal mice Furthermore, killing within phagocytes appears impaired in cortisone-treated animals Thus, slide cultures of infected muscle suggest that more fungi survive within and grow out of polymorphonuclear leukocytes after phagocytosis Other experiments indicate that potassium deficiency altered carbohydrate metabolism impairment of circulating inhibitors of monilia growth and dysfunction of the reticuloendothelial system are not major factors in cortisone enhancement

In summary cortisone enhances experimental *Candida* infections to a far greater degree than infections due to *H capsulatum* or *C neoformans* Enhancement of infection is first generalized This may be related to decreased mobilization of inflammatory cells and to dysfunction of polymorphonuclear phagocytes

The subsequent limitation of progressive infection to the kidney its limited occurrence in normal mice and its uniform occurrence in cortisone treated animals probably can be explained by the ability of the fungus to break into and proliferate within renal tubules a site in which the fungus is protected from host-cellular defense mechanisms

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THE PATHOGENESIS OF 'EPIDEMIC' HISTOPLASMOSIS

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Histoplasmosis has come to be recognized as an infectious disease of significant incidence in the endemic area. Its manifestations are protean and in most cases they mimic tuberculosis clinically, radiologically, and anatomically. Differentiation is possible by demonstration of a rising titer of complement fixing antibodies for *Histoplasma capsulatum* or, preferably, of *H. capsulatum* by microscopy and culture.

The primary infection is caused by inhalation of spore containing dust and usually heals without major clinical symptomatology but frequently with massive calcification. This calcification may produce symptoms many years later.

The development of active disease, as in most infections, depends greatly on the infective dose and virulence of the organism on the one hand and what is called the resistance of the patient on the other. Numerous cases of overwhelming infection with *Histoplasma* have been observed in the so called epidemics of histoplasmosis[1-6]. We have observed several such massive infections in the past two and one half years and find the principles revealed by these cases not only pertinent to the understanding of epidemics but also to understanding the pathogenesis of the disease.

In the first outbreak, which occurred in a rural community just outside of Cincinnati, 3 persons (2 adults and 1 child) had entered an abandoned chicken house and spent several hours cleaning it. There was much dust, and all were exposed to it. In 7 to 10 days both of the adults were clinically ill, but only 1 had severe pulmonary and systemic symptoms. In 14 days the child developed symptoms. X rays of all 3 taken about 5 to 6 weeks after the exposure revealed diffuse miliary and nodular infiltrates throughout both lungs. In addition, the child had prominent hilar adenopathy. All 3 patients recovered after several weeks, 2 with hospitalization and amphotericin B therapy and 1 with no treatment. A high titer of complement fixing antibodies with a negative tuberculin skin reaction and conversion of a negative to a positive histoplasmin skin test reaction comprised the presumptive evidence of histoplasmosis in the child, and the clinical course and history made this diagnosis appear certain in the adults.

Three more cases, 2 in one family, were seen in Hamilton, Ohio and, although the relationship to the source of the organism was not established, the clinical picture combined with rising titer of complement fixing antibodies pointed unmistakably to the diagnosis. One of the 2 cases from the one family was a

12 year old girl. The only male dated the onset of his disease to 2 weeks following his cleaning out a chicken house. The patient C. K., is particularly noteworthy since he cleared his disease markedly, but developed a recurrence 2 years later. At this time a lung biopsy was done and revealed active granu-



FIGURE 1. Milk glass appearance due to extremely heavy pulmonary lesions in child L. T. after exposure. Marked hilar involvement.

lomatous lesions with yeast cells morphologically identical with *H. capsulatum*. The patient was then begun on treatment with amphotericin B and has responded clinically although X ray worsening has appeared to occur temporarily. Subsequent follow up has shown improvement.

Comments and Discussion

The history and X ray findings of the 6 people clearly follow the pattern of massive inhalation of spore containing dust observed in previous epidemics.[1-5] The pathogenesis of the multiple foci would indicate 2 possibilities. One is

that these represent multiple primary foci due to the huge infective dose contained in dust entering the airway The second is that there develops a single primary focus from which hematogenous dissemination occurs The clinical picture and X ray experience undoubtedly point to the former The

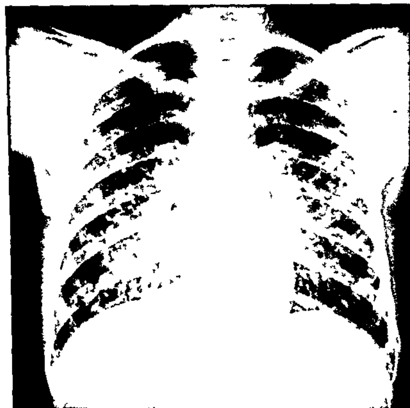


FIGURE 2 Widespread nodular densities in both lung fields and heavy hilar infiltrates patient C K October 1956 (three weeks after exposure)

lesions appear 1 week to 10 days after exposure and are rather uniform in size and rather large when first seen It is much more likely that this represents the appearance of multiple primary foci[6] than multiple hematogenous foci Multiple primary foci of similar appearance exist in dogs (M Straub in preparation) This observation plus the sequence observed in case C K combined with the pulmonary biopsy strongly support this view

We consider the isolated observation of the following case significant because

it represents the terminal stage (calcification) of a massive exposure to spore-containing dust A 36-year old white male was shown in many medical meetings as a classic example of multiple pulmonary calcification supposedly related to mitral stenosis At autopsy hundreds of uniformly calcified round foci 2 to 5 mm in diameter were found We were very easily able to demon

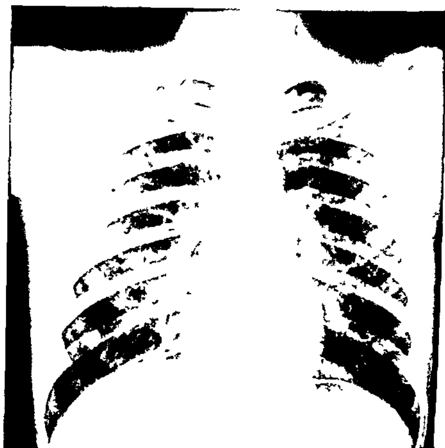


Figure 3 Marked improvement in December 1937

strate morphologically organisms of *H capsulatum* in each of the numerous examined lesions Microscopically all of the lesions showed a similar structure a bony rim with calcification of the center Frequently the bony rim had a cavity filled with bone marrow and the whole structure was surrounded by a considerable area of scar tissue The scar formation clearly indicated that originally a perifocal inflammation had existed Each of these lesions if seen alone would be indistinguishable from ordinary solitary primary foci seen in spontaneous human infections

It therefore can be concluded that in a so-called epidemic the concentration of infectious material in the air is such that numerous simultaneous primary foci are formed in the pulmonary tissue These foci undergo necrosis calcification and even ossification

It was a fortunate circumstance that in the case of C K a biopsy was taken

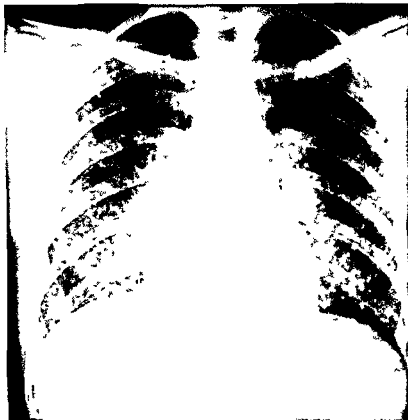


FIGURE 4 Relapse in April 1959

The active lesions in the lungs of this patient can be considered multiple primary foci They represent a developmental stage which terminates in the formation of completely calcified foci with an osseous rim

It is this terminal stage that was found to exist in the lungs of our patient C K who obviously many years after exposure died from cardiac complications unrelated to the infection with *H capsulatum*



FIGURE 5 Gross appearance of biopsy specimen, same patient

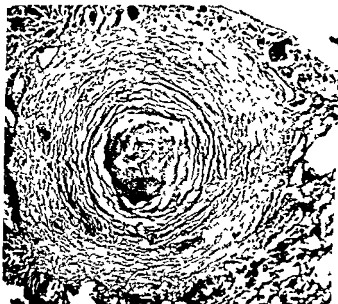


FIGURE 6 The individual foci were well circumscribed but active and yeast cells were demonstrated in all (same patient) Hematoxylin eosin ×25



Figure 7 Multiple calcified foci in lung of patient with mitral stenosis October 19[illegible]
(C K)



FIGURE 8 X ray of inflated autopsy specimen (1953), same patient

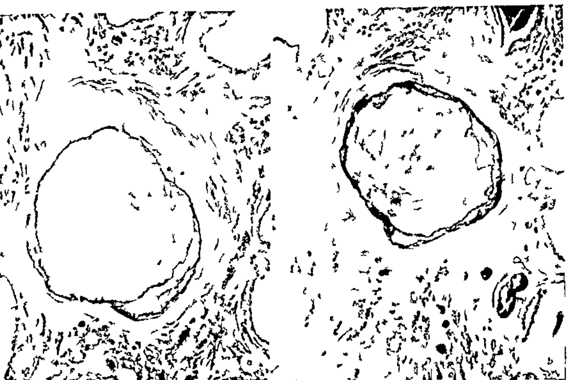


FIGURE 9a and b [illegible] calcified multiple primary foci with osseous [illegible] proved to be histoplasmic in origin [illegible] same patient *Hematoxylin and eosin* Note the abundant scar tissue surrounding the calcified foci ×35

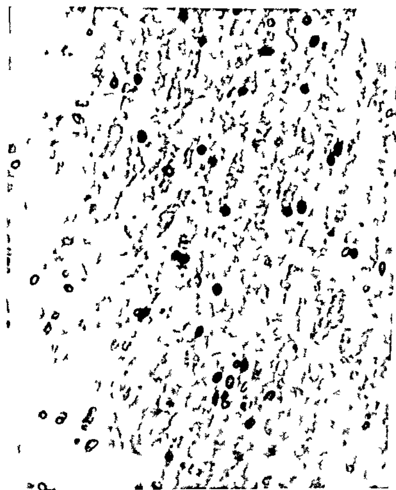


FIGURE 10 Yeast cells 3 to 5μ n s ze we e eas ly demons a ed n he foc G ocott silver sta n same pat ent ×600

TABLE

CLINICAL CASES

Case	Age Sex Race	Clinic	X-ray	Skin test	Complement fixation antigens			
					Dates	Yeast	Mycel	Misc
T F	42 MW	12-23-1957 cleaned chicken house. Weight loss, weakness, tiredness, aching	1946 clear X-ray. 1-26-1958 miliary densities widespread		1-30-1959	Neg	Neg	
L T	28 MW	12-23-1957 cleaned chicken house. Fever, anorexia, cough, weight loss l y 1-1-1959	1-29-1958 miliary nodular densities, severe		1-20-1958	1:128	1:32	Rx with Amphotericin B
L T Jr	2½ MW	12-23-1957 cleaned chicken house. Fever, nausea, weakened by 1-6-1958. Cleared by 1-22-1958	1-6-1958 marked miliary involvement with hilar adenopathy	PPD No. 1 neg.; histoplasmin neg. 1-58; histoplasmin pos. 2-26-1959	1-6-1958 1-28-1958	1:128 1:256	1:32	Rx with Amphotericin B
L H	12 FW	No symptoms, slight fatigue	11-26-1956 hilar adenopathy. 12-24-1956 progression with *LUL* infiltrate. 3-9-1957 LUL infiltrate resolving	PPD No. 1 neg.; histoplasmin *positive*	1-4-1957	1:8	1:32	
B L	24 FW	Weight loss, cough, fever, dyspnea, headache	11-21-1956 *massive nodular* infiltrate. 11-28-1956 progression with conglomeration. 12-8-1956 marked clearing. 1-4-1957 more clearing	PPD No. 2 neg.; histoplasmin positive	12-26-1956 1-23-1957	1:128 1:64	1:8 1:16	Very sick. Rx O_2 prior to chemotherapy
C K	16 MW	Cleaned chicken house. Flulike syndrome cleared in 1 month	*10-20-1956*, marked nodular disease. 12-3-1956 slight improvement. 12-31-1956 more clearing. 12-4-1957 more clearing with calcification. 9-4-1958 increase in size of lesions. 1-5-1959 definite increase. 4-2-1959 marked increase in parenchymal and hilar lesions. 5-6-1959 definite im	*PPD* No. 2 neg.; histoplasmin positive	11-20-1956 12-4-1956 1-10-195[illegible] 2-10-1959	1:64 1:32 1:32 1:64	1:32 1:8 ? ?	Rx with Amphotericin B

Summary

Histoplasmosis is acquired by inhalation of spore containing dust In special circumstances (massive infective dose) when the air becomes densely loaded with spores there occurs a special clinical, radiological, and anatomical picture, consisting of multiple primary foci in the lung The majority of cases recover

Seven examples of multiple widespread densities in the lung are given one definitely proved by biopsy to be active and one shown at autopsy to have multiple healed primary lesions Histoplasma organisms were demonstrated microscopically in both

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THE OCCURRENCE OF *CANDIDA* AND OTHER YEASTS IN THE INTESTINAL TRACTS OF ANIMALS

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Yeasts are extremely widespread in nature They can be isolated from almost any surface soil They are also encountered in rivers and lakes and in the water and mud of the oceans and seas Recently they were encountered regularly even by an Arctic expedition (Kriss 1955) that cultured water samples taken at a distance of about sixty miles from the North Pole

In light of what is known about the nutritional requirements of yeasts this ubiquity is rather surprising In fact the yeasts prefer, and in most cases exclusively use as carbon sources, simple carbohydrates that do not accumulate in appreciable amounts in soil and certainly not in sea water A number of yeast species cannot attack naturally occurring carbohydrates other than glucose, mannose, and fructose Many also use one or more other hexoses, pentoses, oligosaccharides alcohols organic acids, and related compounds, not too many are capable of breaking down starch or inulin, and a few have proteolytic or lipolytic capacity However, the breaking down of compounds such as cellulose or lignin is already beyond the biochemical means of the yeasts Therefore, it seems reasonable, as suggested by Lund (1954), that normally the multiplication of yeasts in soil is transitory or nonexistent The soil would be a reservoir where yeasts survive while awaiting opportunities for invading their natural substrata These natural substrata, including materials such as sweet fruits decaying mushrooms, exudates of trees and flower and tree-visiting insects are rich in simple carbohydrates

Simple carbohydrates are also readily available in the intestinal tract of animals where they are produced in the process of digestion or, as such are already present in ingested food They are also readily available in amino acids which are happily accepted by yeasts as a source of nitrogen and of vitamins of the B group one or more of which are required by a great many species of yeasts There is normally a generous supply of these in the animal digestive tract and it appears quite understandable that several species of yeasts have become adapted to this comfortable environment

Virtually nothing is known of the ecology of marine yeasts Possibly the natural substrata of these yeasts are aquatic vegetables and animals that may supply adequate amounts of simple carbohydrates Recently some preliminary evidence was obtained in my laboratory indicating that the intestinal tracts of fishes may constitute such a substratum

I now propose to consider in some detail the yeasts that occur in the digestive tracts of certain terrestrial mammals and birds Given the ubiquity of yeasts in nature their introduction with food and dirt into the digestive tract of humans and animals must be a daily occurrence Those yeasts that resi[illegible] destructive action of the digestive enzymes will eventually appear alive [illegible] feces, from which they can be isolated However, how can we [illegible]

yeast isolated in this way is an habitual inhabitant of the digestive tract rather than a passer by sufficiently fortunate to escape from a dangerous environment?

A first approach to this problem is a qualitative survey intended to isolate and identify yeasts from not too small a number of specimens of the host species under study The relative frequencies of the yeast species encountered as compared with their occurrence outside the animal body constitute a primary basis for the ecological classification of the intestinal yeasts

In this way the intestinal yeast flora of man and to a much lesser extent of other warm blooded animals have been subjected to study for many years but unfortunately much of the older, and some of the more recent work has been executed with rather unsatisfactory techniques

A first point of great practical importance is the choice of the isolation medium Sabouraud s medium for example is not appropriate because it may lack essential growth factors needed by some true intestinal yeasts Classic Sabouraud s medium prepared with maltose is still worse as many yeasts cannot use this sugar A glucose peptone broth enriched with liver or yeast extract and made selective with antibiotics will suffice The only known yeast that will not grow well in such a medium is *Saccharomycopsis guttulata* an inhabitant of the digestive tracts of rabbits

A second point is concerned with growth temperature Mycologists are very much accustomed to doing their isolation and identification work at room temperature and often this is satisfactory However care should be taken under such circumstance not to conclude—as occasionally happens—that a yeast strain or any other fungus encountered is a saprophyte or even a pathogen of warm blooded animals when the strain is not even able to grow at the body temperature of its supposed host or victim In so far as surveys on the intestinal yeast flora of warm blooded animals are concerned isolations should be done at about 37° C In this way may passers by unable to grow at body temperature are eliminated a priori but there is another important reason for using this temperature A few yeast species such as *Candida slooffii* and *Torulopsis pintolopesii* highly adapted to warm blooded hosts cannot be isolated at room temperature because their minimum temperature for growth is higher

The third practical point that I shall stress is concerned with identification methods That these methods should be sound is self evident However until recently they were far from standardized and were based partly on erroneous concepts At the time yeast taxonomy was still much confused The identification of a yeast strain not belonging to one of a few well known species was sometimes a question of courage rather than knowledge Consequently some of the older work on yeasts associated with man and animals must be regarded with suspicion However even today occasional workers in the field do not seem to be aware of the progress that has been made in yeast taxonomy and identification methods Certainly anyone who wishes to identify yeasts should find it worthwhile to make himself acquainted with the work of modern yeast monographers such as Lodder and Kreger van Rij (1952) in the Netherlands and Wickerham (1951) in the United States

When the results of surveys done on large numbers of specimens of various warm blooded host species are compared it appears at once that certain yeast species are encountered much more frequently than others We may suppose

that the former are true intestinal yeasts, whereas the others are concomitants that happen to be able to grow at body temperature and simultaneously to resist the destructive action of the digestive enzymes

Within the group of true intestinal yeasts, that is, the yeasts for which the digestive tract is a natural habitat, two subdivisions are defined rather sharply obligate saprophytes and facultative saprophytes The former have their natural habitats exclusively in the digestive tracts of animals, the latter may grow under natural conditions also on certain substrata outside the bodies of animals Within the group of obligately saprophytic intestinal yeasts, various degrees of host specificity and nutritional fastidiousness are found, and it would seem that these two characteristics are correlated

S guttulata represents an extreme type This saprophyte of the rabbit's digestive tract although known since 1845 (Remak) has resisted for more than a century all efforts to grow it in pure culture Only recently Parle (1956) and Shifrine and Phaff (1958) succeeded, independently, in doing so The latter two authors made a thorough study of this species and found among other interesting properties that the temperature range for growth of *S guttulata* is rather narrow being limited by the very high minimum temperature of 35° C

Inability to grow at room temperature in a yeast species encountered regularly in the digestive tract constitutes the strongest evidence that the yeast in question is a true obligate saprophyte This inability to grow at room temperature thus far has been found only in two other yeast species both identified in my laboratory

C slooffii (Van Uden and Carmo-Sousa 1957*a*) will not grow below 28° C Although less fastidious than *S guttulata* it is more so than ordinary yeasts We found it to require biotin pantothenate inositol niacin pyridoxine and thiamine, and Cury informed us that a number of amino acids are also essential growth factors for this species Its host specificity is somewhat less pronounced than that of *S guttulata* Whereas the latter seems to be exclusively an inhabitant of the rabbit and probably of the chinchilla with an incidence of nearly 100 per cent, *C slooffii* is a saprophyte of the domestic pig's digestive tract with an incidence of about 50 per cent We have found this species also in African bush pigs (*Potamochoerus choeropotamus*) but not in two other relatives of the domestic pig the wart hog (*Phacochoerus aethiopicus*) and the hippopotamus (*Hippopotamus amphibius*) Neither did we isolate this species from humans, baboons (*Papio cynocephalus*) African pied crows (*Corvus albus*) cattle goats or sheep The type strain however was [illegible] 6 isolated from 6 of 252 horses

The other thermophilic species is *Torulopsis pintolopesii* (Van Uden, [illegible]). This yeast does not grow below 24° C and is slightly less fastidious [illegible] *slooffii* It is a frequent inhabitant of the digestive tracts of rats [illegible] from which it has been isolated by several investigators It seems [illegible] also in other small rodents and in pigeons A number of stra[illegible] C W Emmons from these birds proved to be *T pintolopesii*

Candida bovina a species that I described with Carmo-Sou[illegible] strain isolated from the cecal contents of a cow, takes an inter[illegible] between the thermophilic fastidiou[illegible] highly host-spec[illegible]

phytes and the other obligate saprophytes such as *C albicans*, which grow easily at room temperature on ordinary culture media and can be isolated from a wide range of hosts *C bovina* grows slowly at 20° C It has been isolated from cattle, swine, rodents, and turkeys This does not mean necessarily that these four groups of animals are its true hosts A yeast that is a true saprophyte of certain animal species may occur as a fortuitous concomitant in others In this case also numbers are helpful A single strain of *C bovina* isolated from cattle (Van Uden and Carmo-Sousa, 1957) suggests that *C bovina* is such a concomitant when occurring there, but an incidence of 14 per cent found in swine (Van Uden, Carmo-Sousa, and Farinha, 1958) strongly points to these animal species as natural hosts for *C bovina*

Recently Van der Walt (1957) described as *Saccharomyces telluslris* a yeast that he had isolated from soil Kreger-Van Rij (1958) discovered that this sporulating yeast is the perfect form of *C bovina* On the other hand, Shifrine and Phaff (1958) showed that *S guttulata*, although incapable of vegetative growth below 35° C, will sporulate at room temperature and even at 18° C *These interesting findings permit the conclusion that these yeasts, although* highly specialized for life in the digestive tract of warm blooded hosts, may form spores outside the animal body and survive as such When these spores happen to find their way into the digestive tracts of the proper animal species vegetative growth will start again

We now come to the problem of *C albicans* This species, unlike *S guttulata*, *C slooffii*, *T pintolopesii*, *C bovina, and S tellustris* is not distinguished by a high minimum growth temperature or unusual nutritional fastidiousness Nevertheless it is a true obligate saprophyte of warm blooded animals and it has been isolated from substrata outside the animal body on a few occasions only It should be worthwhile to determine whether *C albicans* survives outside the animal body through its capacity, unique among the yeasts of forming chlamydospores

The host specificity of *C albicans* is of a low degree, and this species has been isolated from a wide range of hosts However, before reporting the findings in the material studied in my laboratory, I herewith introduce the term total *yeast index*

The total yeast index is the quotient of the total number of isolated strains and the total number of the individuals of the same host species sampled This index number may become higher than 1 when more than one yeast species is isolated from a number of individuals In addition to the total yeast index, a percentage number may be used to indicate the proportion of individuals found to harbor yeasts regardless of the number of different species isolated from each of them As the incidence of a single yeast species, for example *C albicans* can never be higher than 100 per cent in a group of host individuals, it can be expressed conveniently in a percentage number

In this way TABLE 1 shows the total yeast indexes, the percentage numbers of positive individuals and the incidences of *C albicans* found in our material

It can be seen at once that under natural conditions big differences exist between the various host species with regard to the occurrence of yeasts in their digestive tracts Not much is known of the factors that may determine the degree of suitability of warm blooded animals for harboring yeasts, but it

gestive tract In Portugal the three primary types of alimentation used in raising pigs showed the following correlation with the occurrence of *C slooffii* (1) most pigs that did not reveal the presence of *C slooffii* had been kept on a green food diet, (2) most pigs with low cell numbers of *C slooffii* throughout the digestive tract had been kept on a mixed diet, including refuse from the human kitchen and (3) most pigs with high counts for *C slooffii* in one or more sites of the digestive tract had been kept on a grain diet chiefly corn Consequently, it seems a promising proposition to direct more research in this direction that is the relationship between diet and the intestinal yeast flora

In so far as *C albicans* and its importance in pathology are concerned more knowledge of the factors influencing its occurrence in the normal digestive tract is desirable as there is evidence that the digestive tract is the base from which *C albicans* launches most of its disease producing attacks The best example studied is yeast vulvovaginitis The question has been raised where may yeasts harmless or not enter the vagina? Yo Bwan Hie (1954) in a well-conducted study was able to show that feces are the principal source of infection and reinfection in yeast vulvovaginitis Magara *et al* (1955) considered the urinary tract also as a reservoir from which the vagina might become infected and reinfected Together with Meleiro de Sousa I studied this problem in Portuguese women Correlation studies were done with the two species that in Portugal are more frequently isolated from the vagina *C albicans* and *T glabrata*

Of 55 women with positive vaginal cultures for *C albicans* 76 per cent had this yeast also in the feces In a control group of 170 women with negative vaginal cultures only 25 per cent were positive for *C albicans* in the feces With *T glabrata* a similar situation was encountered Eighty two per cent of 21 women with positive vaginal cultures for *T glabrata* were found to harbor this same species in the digestive tract whereas only 11 per cent in a control group of 204 women had positive fecal cultures for *T glabrata*

Still more pronounced was the correlation between the presence or absence of yeasts in the vagina and their simultaneous presence or absence in the urinary tract Accordingly 54 per cent of 33 women with positive vaginal cultures also had positive urine cultures Of a control group of 91 women only 2 per cent had yeasts in the urinary tract Therefore it appeared that most women whose vaginas were infected with *C albicans* or *T glabrata* harbored simultaneously the same species in their digestive and their urinary tracts

When the women were cleared of vaginal yeasts by local vaginal treatment cultures were done repeatedly from urine and feces and it was found in all cases that yeasts had disappeared spontaneously from the urinary tract within a few days whereas the occurrence of *C albicans* or *T glabrata* in the digestive tract was not influenced by the disappearance of these yeasts from the vagina This strongly suggests that the digestive tract is an important source of infection in yeast vulvovaginitis whereas the urinary tract is invaded only secondarily from an infected vagina

Finally I shall briefly discuss the kind of information we may obtain when intestinal yeasts are studied by the use of quantitative techniques

In most cases intestinal yeast populations are not sufficiently dense to allow

fowls, and turkeys are natural hosts of *C albicans* Most reports give percentage numbers between 20 and 30 per cent In a group of 225 Portuguese women we isolated *C albicans* from the feces of 38 per cent of the individuals which is of about the same order The fact that, bird species other than fowls and turkeys may also constitute suitable hosts for *C albicans* is shown by our findings in African pied crows 18 per cent of which were positive for this yeast Also certain members of the pig family, including domestic swine African wart hogs and African bush pigs were on the higher side with regard to the incidence of *C albicans* However, even in animal groups with a very low total yeast index *C albicans* was found From 503 sheep only 34 yeast strains were obtained but of these 26 were *C albicans* Strangely, I found rather low incidences in 2 groups of East African natives In a group of 100 men on a low animal protein diet only 12 per cent were found positive for *C albicans* In another group of 102 men on a high animal protein diet the incidence of *C albicans* was still lower—only 4 per cent It is well known that Americans of African ancestry are certainly not less prone to *C albicans* invasion that Americans of European ancestry Race may not be a satisfactory explanation for the low *C albicans* incidences found in my two groups of East African men

This brings us to an interesting problem When a certain animal species apparently is a suitable host for a certain microorganism for example *C albicans* why should only a proportion of the specimens belonging to this host species actually harbor the microorganism in question? Nothing is known of the importance that individual genetic differences may have for the host *C albicans* relationship but some factors that influence the capacity of humans to support growth of *C albicans* are well known to medicine pregnancy diabetes and treatment with broad spectrum antibiotics are some of these Another point to consider is the chance of infection However given the high incidence of *C albicans* in humans every individual probably introduces *C albicans* quite frequently into his digestive tract Once again a factor of real influence may be the type of diet The group of Africans with the incidence of only 4 per cent of *C albicans* and the relatively low total yeast index of 0 79 had a high daily intake of animal proteins in the form of dried fish and game The group with the *C albicans* incidence of 12 per cent and the total yeast index of 1 30 lived almost entirely on a vegetarian diet I do not feel it justifiable to conclude from this meagre evidence that the type of diet influences the incidence of *C albicans* in the digestive tract but there is other evidence that points in the same direction

Rolle and Mehnert (1957) found *S guttulata* regularly in the stomachs of rabbits and chinchillas as long as the animals were on an uncooked vegetarian diet On the contrary in the stomachs of rabbits and chinchillas on a milk diet no cells of *S guttulata* were encountered

In my laboratory some evidence was obtained that the occurrence of *C slooffii* in the digestive tracts of swine is influenced by the type of food on which the pig lives As already related *C slooffii* occurred in the digestive tracts of about 50 per cent of the swine studied by us In one group of 57 swine we gave attention to the type of diet on which the animals had been kept which in most cases is recognized easily from the aspect of the contents of the di

gestive tract In Portugal the three primary types of alimentation used in raising pigs showed the following correlation with the occurrence of *C slooffii* (1) most pigs that did not reveal the presence of *C slooffii* had been kept on a green food diet, (2) most pigs with low cell numbers of *C slooffii* throughout the digestive tract had been kept on a mixed diet, including refuse from the human kitchen, and (3) most pigs with high counts for *C slooffii* in one or more sites of the digestive tract had been kept on a grain diet, chiefly corn Consequently it seems a promising proposition to direct more research in this direction that is, the relationship between diet and the intestinal yeast flora

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Finally I shall briefly discuss the kind of information we may obtain when intestinal yeasts are studied by the use of quantitative techniques

In most cases intestinal yeast populations are not sufficiently dense to allow

a quantitative evaluation by the direct microscopic examination of intestinal contents *S guttulata* is an important exception By the use of this simple semiquantitative method several investigators found that *S guttulata* is adapted to life in the rabbit's stomach There the vegetative cells propagate by budding On passage through the digestive tract growth stops or is considerably reduced For quantitative studies of other intestinal yeast species plate counts may be used We conducted such studies with 57 pigs One gram samples of intestinal contents were taken from the stomach, from three sites in the small intestine, from the cecum, and from the rectum

In 27 animals *C slooffii* was found in one or more sites in the digestive tract in numbers ranging from less than 300 to 9,000,000 cells/gm of wet content Generally, the cell numbers of *C slooffii* varied considerably from site to site in the digestive tract of the same animal The trend was an increase of cell numbers in the oral anal direction The highest cell number found in each animal was obtained more often from rectal contents than from other sites of the digestive tract, and the second highest counts were most frequent in samples from the cecum

Although *C slooffii*, both qualitatively and quantitatively, was the prevailing yeast, in many cases other yeast species were found in appreciable numbers However, only from 3 of the 57 animals were counts of more than 100 cells of *C albicans* per gram wet intestinal content obtained On the other hand, 3 species, *C krusei*, *Pichia membranaefaciens*, and *Saccharomyces cerevisae* appeared, their cell numbers ranging from more than 100 to more than 2 million/gm wet content in about 12 per cent of the animals

Since these three yeasts are frequently encountered in food, their frequent occurrence in the digestive tracts of many warm blooded animal species therefore does not constitute in itself proof that they would be anything more than mere concomitants

Even their occurrence in the digestive tract with high cell numbers does not constitute such proof, as the ingested food might have been heavily infested However, as in *C slooffii* and also in the three yeasts just mentioned, we found a cell number increase in the anal direction, with highest counts in the rectum Therefore, it must be concluded that the latter multiply actively while passing through the digestive tract and therefore must be classified ecologically as facultative saprophytes of the digestive tracts of warm blooded animals

Summary

The yeasts, although widespread in the soils and the seas seem adapted primarily to substrata where simple carbohydrates are readily available The digestive tract of warm blooded animals is one of these

Intestinal yeasts may be recognized through qualitative and quantitative surveys Essentials for successful surveys include an isolation medium sufficiently rich in growth factors to support fastidious species such as, *C slooffii*, an isolation temperature appropriate for thermophilic yeasts, and soundness of methods of identification

The suitability of the digestive tract for yeasts depends on the host species and on individual factors, including type of diet The following total yeast

indices (number of strains, number of individuals) have been found in my laboratory (the incidence of *C albicans* is indicated in parentheses) African bush pigs (*Potamochoerus choeropotamus*), 2 00 (11 per cent), Portuguese women, 1 48 (38 per cent), African pied crows (*Corvus albus*), 1.34 (18 per cent), baboons (*Papio cynocephalus*), 1.31 (8 per cent), East Africans on a low animal protein diet, 1 30 (12 per cent), swine, 1 16 (9 per cent), East Africans on a high animal protein diet, 0 79 (4 per cent), horses, 0 58 (4 per cent), African wart hogs (*Phacochoerus aethiopicus*) 0 56 (22 per cent), cattle, 0 52 (0 per cent), hippopotami (*Hippopotamus amphibius*), 0 37 (0 per cent), sheep, 0 07 (4 per cent), and goats, 0 06 (1 per cent)

Yeasts occurring in digestive tracts may be divided into three ecological groups (1) obligate saprophytes, (2) facultative saprophytes, and (3) passers-by

Certain obligate saprophytes show a high degree of host specificity *S guttulata* is adapted to rabbits, *C slooffii* to swine, *T pintolopesii* to mice and other small rodents Other true intestinal yeasts seem slightly better adapted to certain host species than to others *C albicans* is encountered more frequently in humans and certain birds than in other host species but was found with low incidences in two groups of East Africans

Obligate saprophytes may survive outside the animal body as spores, whereas the other intestinal yeasts are more or less capable of growth outside the animal body

The type of diet seems to influence considerably the density and the composition of intestinal yeast populations

The human intestinal tract constitutes the principal source of vaginal infection from *C albicans* and other yeasts, whereas the urinary tract is infected secondarily from an infected vagina

Within the digestive tract different species may be adapted to different sites Whereas *S guttulata* has a preference for the stomach, *C slooffii*, *C krusei*, *P membranaefaciens*, and *S cerevisiae* show a cell number increase in the anal direction the highest counts being obtained from the rectum

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EPIDEMIOLOGY OF THE DERMATOPHYTOSES SOURCES OF INFECTION MODES OF TRANSMISSION AND EPIDEMICITY

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The dermatophytes and the diseases that they produce may be divided into several groups on the basis of sources of infection host preference, and epidemicity

As might be expected human infections in rural areas are commonly acquired from domesticated or wild animals Such infections usually evoke considerable tissue reaction in the host and are characterized by suppuration kerion formation and allergic phenomena that appear to be responses to fungi not well adapted to existence in human tissues

Until recently we have had little knowledge of the prevalence of ringworm infections of animals or information regarding the frequency of their transmission to humans In the past few years however several epidemiological studies and surveys such as those conducted in England by Ainsworth and Austwick [1] La Touche [2] Gentles and O Sullivan [3] and McPherson [4] in Yugoslavia by Ožegovic and Grin [5,6] and in the United States by the Communicable Disease Center in Atlanta Ga [7,8] have been carried out The results of these studies have shown the high prevalence of ringworm in animals and have given considerable evidence on the manner in which these infections are transmitted to humans

The individual dermatophyte species have varying degrees of host specificity Some are primarily animal pathogens These are the so-called zoophilic fungi Although animals appear to be the natural hosts for this group of fungi such infections frequently are transmitted to humans Six dermatophyte species that commonly cause ringworm in animals have been found to be important in relationship to human infection These include *Microsporum canis M distortum Trichophyton verrucosum T mentagrophytes* (var *granulare*) *T equinum* and *T gallinae*

Human infections due to *M canis* are common in both rural and urban communities and there is little doubt that most cases are of animal origin This fungus is the most common cause of ringworm in cats and dogs In the Communicable Disease Center survey 325 feline specimens yielded dermatophytes Of these 318 or 97 8 per cent were *M canis* Of 495 canine specimens 329 or 66 5 per cent were *M canis* Cats and dogs appear to be the most important source of *M canis* infections in man

Human infections due to *T verrucosum* are confined almost entirely to the rural population This fungus causes the vast majority (98 to 1[illegible] per cent in some areas) of ringworm infections in cattle [1,5] Most human infections are easily traced to direct contact with ringworm infected cattle [illegible] A number of cases of indirect transmission have been reported also [illegible] In some instances it appears that infected cattle hairs have been carried on the clothing of the farmer into his home where children, including infants have become infected indirectly [11] Walker[12] has shown that *T verrucosum* remains [illegible]

infected hairs in the laboratory for 15 months, and she has demonstrated the presence of *T. verrucosum* infected hairs on a scratching post used by cattle infected with ringworm.

Infections due to *T. mentagrophytes* (var. *granulare*) occur also largely among the rural population of the United States.[11] However, in contrast to ringworm due to *T. verrucosum* that usually is traced to contacts with infected cattle the source of *T. mentagrophytes* infections often is not easy to discover. Although *T. mentagrophytes* has been reported as a cause of ringworm in cattle and horses,[18, 19] results of recent surveys indicate that such infections are relatively rare.[1, 8, 20] In the Communicable Disease Center survey, *T. mentagrophytes* was isolated only once from a cow and once from a horse. Ainsworth and Austwick[1] recorded only one equine case and no cattle infections in their survey in Great Britain. Infections in cats and dogs appear to be somewhat more common. In the Communicable Disease Center survey, 3 isolates were made from cats and 45 from dogs (this latter constituted 9.1 per cent of 495 canine hair specimens).

In contrast to this rather low prevalence of *T. mentagrophytes* infection among domesticated animals, there is considerable evidence that infections are common among rodents. The disease often is seen in the laboratory guinea pig and mouse.[21-27] It also has been shown that the disease is not uncommon among wild rodents.[28] It is noteworthy that *T. mentagrophytes* is recovered frequently from animals even though they do not have skin lesions or hair invasion that can be detected either grossly or microscopically.

The fact that rodents are commonly infected by this fungus suggests that certain areas of farm premises frequented by rodents, especially feed bins and barns, may be contaminated by spores and infected hairs shed by these animals. It is felt that rodents play an important role in the transmission of *T. mentagrophytes* infections in rural areas. A number of human outbreaks have in fact been reported as occurring following the handling of grain and straw contaminated by infected mice.[29-31]

Human infections due to *M. distortum*, *T. gallinae*, and *T. equinum* are rare. *M. distortum* was first described by Di Menna and Marples in 1954[32] as the cause of tinea capitis among 12 children in New Zealand. These authors were unable to determine the source of the infection. In 1957 Kaplan *et al.*[33] described the isolation of *M. distortum* from 4 monkeys (3 in Iowa and 1 in Florida) and 1 dog in the United States, and gave evidence for 6 possible human contact infections. Recently a human infection derived from a monkey was reported by Brooks *et al.*[34] in Washington, D. C. It is interesting that this monkey came from the same pet shop in Des Moines, Iowa where the first monkey infections had been found in the United States. All of the monkeys had apparently originated in Central America.

With respect to *T. equinum* and *T. gallinae*, two zoophilic fungi that may infect man, these fungi are almost specific for the horse and gallinaceous birds, respectively, and only rarely produce infections in other animals or man.[20, 35] In the case of *T. equinum* the specificity appears to be based on a physiological requirement for nicotinic acid or its precursors that are available in equine hair, but not in the hair of other animals.[20]

Ringworm of those parts of the body covered by clothing, which includes the

feet in those who wear shoes appears to be due almost entirely to fungi of human origin These so-called 'anthropophilic fungi' include *T mentagrophytes* (var *interdigitale*), *T rubrum*, and *Epidermophyton floccosum*

T mentagrophytes (var *interdigitale*) represents the downy or fluffy colonial form of this fungus It is isolated only rarely from infections in animals, but is associated commonly with chronic tinea pedis in the temperate zones of the world Rare instances of *T rubrum* infections in animals have been reported [illegible] In one of these cases, in which *T rubrum* infection occurred in a dog there was evidence that the disease had been transmitted from the owner to his dog as he had a habit of rubbing the dog s back with his infected feet *F floccosum* has never been reported from lower animals

T mentagrophytes and *T rubrum* are by far the commonest causes of tinea pedis in this country and in many other parts of the world The disease produced is usually of a chronic nature, with little tissue reaction The heavily keratinized soles and the intact skin of the feet offer little opportunity for the fungi to penetrate deeply enough to obtain the nutrition that appears to be essential for effective parasitism In fact in many individuals particularly during the winter months when the feet are cool and dry the dermatophytes exist on the tissues of the feet in an almost saprophytic state apparently deriving nutrition from the epithelial debris on the skin surfaces and giving no visible evidence of infection Symptomatic infection of the feet usually is observed first in the toe webs In these areas conditions of warmth and humidity and macerated skin allow the fungus the conditions necessary for it to proliferate From these areas, the infection may spread to an injured nail bed or over large areas of the feet In tropical climates the infection may spread onto the body The opportunity to invade the lanugo hairs offers further opportunity for the fungi to produce disease

The relationship between the downy form of *T mentagrophytes* isolated from chronic tinea pedis and the granular form isolated from suppurative ringworm acquired from animals is a most interesting one My experimental studies[illegible] using monospore isolates of *T mentagrophytes* have shown that the downy and granular forms indeed represent two cultural forms of the same species The downy type which apparently has low virulence for man also shows low virulence for the guinea pig and the dog Virulence however may be enhanced by serial passage in guinea pigs and this increased virulence is accompanied by a morphologic change to a granular type culture similar to that seen in spontaneous animal ringworm The same transformation may also be observed in man when conditions of heat and humidity and communal life as experienced among military personnel in tropical areas are provided This is demonstrated quite clearly in the epidemiological studies by Sanderson and Sloper [illegible] A survey by Walker[illegible] had indicated that *T interdigitale* or the downy type culture of *T mentagrophytes* was isolated consistently in all cases of ringworm of the feet which she had observed in Great Britain However when groups of army men were sent from England to Malaya and Hong Kong Sanderson and Sloper reported that not only was the incidence of ringworm of the feet greatly increased but the cultures isolated in the majority of the cases were of the granular type that they identified as *T mentagrophytes* Correlated with the change of cultural type were an increased severity of the lesions and a large

number of cases of *T mentagrophytes* infections of the body, face, and scalp The lesions on these latter areas frequently showed deep involvement of the hair follicles and suppurative reactions Sanderson and Sloper presented evidence that these body lesions probably had been acquired largely from active foot infections that had spread from the patient's own feet In a number of cases, '*T interdigitale*' was isolated from a primary infection, but later *T mentagrophytes* was isolated from recurrent lesions These investigators show quite clearly that the climatic conditions in southeast Asia that caused intense sweating especially in new arrivals were an important factor in increasing the incidence of ringworm disease among these men In addition, the conditions of army life—communal living poor bathing facilities, interchanges of boots, and borrowing of socks probably increased the spread of the infections from man to man The authors suggest that "if a transformation of '*T interdigitale*,' present on the feet of these men when they left the United Kingdom, into *T mentagrophytes* were postulated, the rapid spread of trichophytic ringworm in this unit could be more easily explained'

The experimental proof that such a transformation can occur does aid in explaining this phenomenon Conditions more favorable for the growth and transmission of the fungi from person to person permitted this dermatophyte to invade the tissues to a greater extent than previously and to produce more tissue reaction Concurrently with the production of more active infections, the fungi became granular

T mentagrophytes therefore, cannot be classed as either zoophilic or anthropophilic but rather should be recognized as having two forms These are the downy form (var *interdigitale*) associated with chronic tinea pedis and the granular form (var *granulare*) associated with animal infections, or human infections of either animal or human origin that have become acute and progressive

It would appear that, besides conditions of reduced resistance of the host and favorable conditions of warmth and humidity, symptomatic and progressive ringworm may be caused by virulent strains that have been developed selectively wherever better nutrition is afforded by opportunity for invasion deep into the skin or hair follicles It has been well established that the dermatophytes seek the areas of newly forming keratin deep in the skin or hair follicles for their maximum development

In many areas of the world, *T rubrum* is the common cause of tinea pedis and tinea corporis In India, where it is the most important ringworm agent, the disease among the large low income group which does not wear shoes, is confined almost entirely to the covered parts of the body Most of these infections, although covering large areas, are chronic in nature, which suggests that this fungus has become well adapted to existence in human tissues However, a tropical climate and crowded living conditions have increased the prevalence of this fungus disease in the tropical areas of India According to a recent study of ringworm infections in Bombay by S C Desai (*personal communication*) there is considerable evidence for familial transmission of *T rubrum* infections Studying the family contacts of 25 individuals with *T rubrum* infections Desai found an infection rate of 41 per cent in the 25 families concerned

This high rate of infection among family contacts suggests that *T rubrum* infections are more contagious than many other diseases

I have had an opportunity to study a number of the T rubrum isolates from India Some of the strains were similar to the white fluffy isolates that are commonly obtained from tinea pedis in the United States However, many of the strains formed the heaped and folded type of colony with a velvety surface and deep rose to purplish red pigmentation on the front as well as on the *reverse of the colonies* This type is usually described as the granular form of *T rubrum* and is characterized by the production of many micro- and macroconidia Desai has demonstrated that experimental human infections are easily accomplished with his Indian strains In my opinion this not only reflects (1) susceptible individual, possibly due to poor nutrition and (2) favorable conditions of warmth and humidity, but it also suggests that there may be an increased virulence in the Indian strains resulting from frequent human passage and favorable conditions for parasitism Experimental infec tions are usually *difficult to produce with strains isolated in the United States* [43]

Experimental evidence that animal passage may increase the virulence of *T rubrum* has been shown recently by Lattridge[44] who demonstrated by serial passage on the chorioallantoic membrane of the developing chick that the viru lence of *T rubrum* may be enhanced gradually Accompanying this increase *in virulence there was a morphologic change from the fluffy to the granular* type

Studies of *T rubrum* infections in families by English[45] in Great Britain and by Rothman[46] and Many *et al*[47] in the United States give further evidence for familial transfer of *T rubrum* infections In the study by Many *et al*[47] the relation of exposure and infection of *T rubrum* within 30 household groups representing 127 people was presented The study revealed that multiple infec tions occurred in 37 per cent of the 30 household contacts examined Long *periods of exposure in some instances 6 months to a year appeared to be neces*sary in the spread of many of these infections among family contacts

The mode of transmission of tinea pedis from one individual to another has long been a source of conjecture as there is little evidence of direct contagion Studies by Baer and Rosenthal and their associates[48, 49] suggested that tinea *pedis is not a transmissible disease in the usual sense and that the environment* does not play an important role in acute outbreaks These authors based their conclusions on the facts that (1) dermatophytes were isolated from normal skin (2) feet placed in suspensions of dermatophyte cultures did not become infected and (3) they had poor success in the isolation of dermatophytes from the environment

It is my belief that these authors did not create the proper conditions for producing infections and that they did not use adequate procedures for the isola *tion of dermatophytes from the environment Ability to produce experimental* infections depends upon many factors including conditions of warmth and hu midity opportunity for the fungi to penetrate the skin virulence of the fungus strain and individual susceptibility of the host

By the use of selective media such as the Cycloheximide Medium[51] and special collecting techniques such as developed by Gentles[52] many isolations

of dermatophytes from floors, shower stalls, foot baths, and from socks and shoes have been made.[50,51,52] The importance of socks and shoes as a source of infection or reinfection has been stressed by Ajello and Getz[3] and Broughton.[54] There is considerable evidence that, once the disease has been acquired, it may be maintained throughout a lifetime with periods of remission and exacerbation.

Recent studies by Trusman and Vanbreuseghem,[56] Gentles and Holmes,[57] and English and Gibson[55] have demonstrated the importance of the following in the epidemiology of tinea pedis.

(1) Age of the host is probably not a factor. The increase in the incidence of infection with age is probably a reflection of increased opportunity for infection rather than increased susceptibility.

(2) The high rate of infection in communal bathers, as compared to those who bathe only at home, gives good evidence that cross infection occurs and is an important factor.

(3) The fact that the rate of infection varies widely in different schools and in groups of individuals using different baths or swimming pools gives further evidence for the epidemicity of this disease.

(4) The percentage of infected individuals can be correlated indirectly with the amount of washing and cleaning of the swimming pool or bathing environment that they regularly use.

That the incidence of tinea pedis in institutionalized children may be very high has been reported by Spoor and Yang.[58] The development of outbreaks of tinea pedis in schools and institutions may be based on both favorable climatic conditions for infection and heavy contamination of the environment. Severe outbreaks may also reflect the development of virulent strains that have been selected by frequent transmission from one individual to another.

In addition to the evidence for transmission of infections, the simple fact that the fungi that cause tinea pedis do not have a saprophytic existence in nature indicates the necessity for either human or animal sources of infection.

With regard to ringworm of the scalp due to the anthropophilic species *M. audouinii*, *T. tonsurans*, *T. violaceum*, and *T. schoenleinii*, there is little doubt as to the source of infection or their epidemicity. These species appear to be highly adapted to existence in human tissues and have been found only rarely in lower animals. To my knowledge *T. tonsurans* has never been isolated from lower animals. The epidemiology of tinea capitis due to these organisms has been treated extensively by a number of workers and will not be discussed here.

Besides the zoophilic and anthropophilic fungi, a third group, the geophilic dermatophytes, remains to be discussed. Two dermatophyte species, *M. gypseum* and the recently described *Keratinomyces ajelloi*,[59,60] exist as saprophytes in soil. Infections of man by *M. gypseum* are quite rare.[61] Animal infections appear to be more common.[8] There is considerable evidence to show that infections in both animals and man are acquired directly from the soil. The higher prevalence in animals is due, no doubt, to their closer contact with soil.

It is interesting to note that soil isolates of *M. gypseum* have very low virulence compared to strains isolated from animals or man. It would appear that either only particularly virulent strains have the ability to produce infections in animals or humans, or that once a strain of low virulence has become parasitic,

due to low resistance of a particular host or opportunity for invasion afforded by an injury to the skin its virulence becomes enhanced and infections may then be transmitted readily to other hosts This would appear to account for the *M gypseum* epidemics that have been reported among horses[20] It might also explain the outbreak among nursery workers in England reported by Whittle[43]

The epidemiology of the dermatomycoses involves many factors source of infection, virulence of the infective agent, favorable conditions of the environment such as warmth and humidity and abrasions of the skin, and individual host susceptibility

In this presentation only a few of these factors have been discussed, however, all are undoubtedly important in the epidemiology of this disease

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NATURAL AND EXPERIMENTAL EPIDEMIOLOGY OF HISTOPLASMOSIS*

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The epidemiology of histoplasmosis has been the subject of many investigations since 1946 There are several postulations as to the natural environment and modes of transmission of *Histoplasma capsulatum* Furcolow, in his *Recent Studies On The Epidemiology of Histoplasmosis*,[1] adequately presents these views, although not to the complete satisfaction of all epidemiologists

Noteworthy contributions to the epidemiology of histoplasmosis have resulted from recent investigations Endemic areas with high incidences of skin test sensitivity to histoplasmin have been discovered in Georgia and Michigan These areas are of epidemiological interest in that they are urban locations and situated in areas where the surrounding histoplasmin sensitivity is low In addition to these two localities where histoplasmin sensitivity and lung calcification have been studied, two epidemics of human histoplasmosis have occurred in Wisconsin and Missouri In each of these epidemics the source of *H capsulatum* proved to be within the city limits and not associated with enclosures

In northwestern Georgia a high frequency of pulmonary calcifications was found in routine X ray films These findings were remarkable in that many of the patients showed sputum negative for tubercle bacilli, and some failed to react to tuberculin Such observations have been relatively common in highly endemic areas of systemic mycoses, especially histoplasmosis However, Whitfield and Murray counties of Georgia are not considered to be in the highly endemic area of this fungus, but may be considered to be on the 'fringe area' For these reasons, a great deal of interest developed, and a search was undertaken to determine the cause of the pulmonary calcifications In March 1957, Edwards *et al*[2] instituted a skin test survey in 6 public high schools in this area Approximately 90 per cent of the students (2111) in these schools received tuberculin, histoplasmin and coccidioidin skin tests The most surprising result was that reactors to histoplasmin ran high in the individuals tested In addition, there was a definite reversal in the usual ratio of rural urban histoplasmin reactors The rural population has generally been considered the most highly sensitized group Students living in the urban area of Dalton, Ga , Negro as well as white, had a sensitivity rate of approximately 55 per cent as compared with a rate of 22 per cent for the rural students The high frequency of histoplasmin reactors among students attending the two urban schools, as compared to the lower number of reactors in the four rural schools suggested a source of inoculum within the urban area of Dalton The authors postulated that contact with a source within the city of Dalton could account for many, if not most, of the histoplasmin reactors found among the rural residents, also, that this source has been present for several years

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A similar high incidence of histoplasmin reactors was discovered in Milan, Mich by Whitehouse et al These investigators were particularly interested in the differential diagnosis of the increasing number of intrathoracic lesions In this southern peninsula area of Michigan the number of tuberculin reactors was 2 0 per cent or less, while the number exhibiting histoplasmin sensitivity was calculated to be 10 to 20 per cent However nearly 90 per cent of the school children studied in Milan were reactors to histoplasmin by the age of 14 In addition to this striking finding 61 5 per cent of the townspeople proved to be reactors to histoplasmin In comparison the population tested in 5 other cities in the area yielded a histoplasmin sensitivity of 2 5 to 10 7 per cent These findings like those of Dalton would strongly suggest a source or sources of inoculum within the city of Milan Here too there is a reversal of the usual rural urban sensitivity to histoplasmin

In the article Walworth Wisconsin Epidemic of Histoplasmosis Wilcox et al have presented a classic in the epidemiology of the disease The epidemic resulted from activity associated with the construction of a new home on a vacant lot in the residential area of the city Nineteen individuals became ill after being exposed for varying periods of time to the source of inoculum These illnesses ranged from mild to severe with an incubation period of 7 to 16 days The nature of the outbreak was such that the investigators were able to determine the exact date and extent of exposure of each individual

There are many interesting points to this epidemic but the failure to ascertain a history of exposure of the lot to chicken enclosures or bird droppings is a significant one Also the fungus was isolated from soil samples collected in the front of the lot and from two scalene nodes It is noteworthy that *H capsulatum* was isolated from soil samples from the front of the lot only The *pH* of the soil was determined and the positive isolations were from samples having a *pH* of 6 0 and 6 55 Negative samples were obtained from the rear of the lot where a higher *pH* prevailed The organism was isolated from collections made in September October December and April

The Walworth epidemic occurred in a nonendemic histoplasmosis area since only 11 4 per cent or less of the population were reported to be histoplasmin reactors In the study of this epidemic the residents of Walworth Village were found to have a much higher histoplasmin reactor rate (15 7 per cent compared to 4 3 per cent) than the rural residents

Another epidemic of histoplasmosis occurred among Boy Scouts in Mexico Mo The site of the epidemic was an eleven acre plot within the city The property was made available to the city for a historical museum At the time of the transaction there were several trees on the property and considerable undergrowth had developed in recent years It was the removal of the latter growth in which the Boy Scouts were engaged that led to their illness Considerable amounts of bird droppings were present at some locations on the site when the clearing activity took place

Since the location is in the endemic area for histoplasmosis skin test sensitivity was known to be high However of the Scouts who worked on the property 97 per cent proved to be histoplasmin reactors whereas among the Scouts who did not work on the property only 40 per cent were reactors Comparing the sensitivity of individuals in the same age range in the high school of Mexico

Mo , the non Boy Scouts showed a reaction rate of 65 per cent and the girls 48 per cent

Fifty two sera were collected from the Boy Scouts who had worked on the property Thirty-one of these were positive (60 per cent), whereas only 6 of 27 (22 per cent) sera from Scouts who did not work on the property showed positive titers A similar observation was made in regard to positive X ray findings Twenty six of 57 (46 per cent) of those working on the property and 10 of 34 (29 per cent) of those who did not work on the property had positive X ray findings

To date 38 isolations of *H capsulatum* have been made from samples collected on this eleven acre site

The complete epidemiological, serological, and X ray findings of this epidemic will be reported by my colleagues at the Kansas City Field Station at an early date

In summarizing these investigations, there are some points that are similar and others that are dissimilar The most remarkable finding was that the source of the inoculum in each instance was within the environs of the particular city In addition, the histoplasmin sensitivity was greater in the urban than in the rural residents This is a distinct reversal in the usual ratio of rural to urban reactors In the Mexico, Mo outbreak, however, the comparison must be limited to those who worked and those who did not work on the site This is particularly important since this city is in an area of high histoplasmin sensitivity Another important observation was that, in so far as possible, no association could be made with chicken or other bird droppings in the foci in Georgia, Michigan, and Wisconsin On the other hand, a distinct correlation can be made in the Missouri epidemic Similar findings were reported in X ray films, and a greater percentage of reactors was found in boys than in girls in all of the studies

The finding of foci of inocula in urban areas does not invalidate the usual observations that *H capsulatum* is frequently associated with chicken enclosures or with other sources of bird droppings To rule out such an association the history of the area over a long period of time must be available It is possible that such an enclosure may have been present but usually this can be ascertained However, contamination by migratory birds may be more difficult to determine Contamination could have occurred many years previously, and the fungus established itself in such foci at that time One must recall that *H capsulatum* does not require only this environment for growth, development, and survival Laboratory investigations have shown that it will thrive on several different types of substrata, providing the humidity and temperature are conducive for growth From all indications *H capsulatum* is a common soil organism and will flourish only in environments that fulfill its minimal or optimal requirements It is obvious that this must have been the case in all of the foci in the studies reviewed

To compare naturally occurring histoplasmosis laboratory studies have been completed that simulate those existir nature It is these contributions that warrant additional discussion t

Reviewing the above and sever al epidemics of histoplasmosis, it is clear that all that infl pment of an epidemic may be

most difficult to determine. In comparison, laboratory epidemiology can be studied under precisely controlled experimental conditions. For these reasons a long range program has been instituted in which studies covering many facets of airborne transmission of systemic mycoses will be investigated.

Sufficient evidence has been reported in recent years to show quite conclusively the importance of airborne transmission in histoplasmosis. *H. capsulatum* was first isolated from the soil by Emmons in 1949. Since that time the fungus has been recovered with great regularity from nature in the endemic area of histoplasmosis. The organism has been isolated from the air and investigations have shown that the mycelium of many isolates forms an abundance of microconidia having a diameter of 5μ or less. The size of these spores is significant since it has been shown that particles larger than 5μ rarely reach the alveoli of the lungs. The respiratory tract seems to be the major portal of entry of the organism under natural conditions. Therefore, the route of inoculation for the study of sensitivity as well as immunological or pathological manifestations elicited by *H. capsulatum* in experimental animals should simulate naturally occurring histoplasmosis.

Sensitivity to histoplasmin has generally been attributed to a past or present exposure to *H. capsulatum*, although cross reactions with related systemic fungi do occur and must be considered in diagnostic or epidemiological studies. There have been some indications that a positive histoplasmin skin test indicates increased resistance to disease. Tissue sensitivity to histoplasmin has been demonstrated in infected laboratory animals. Many investigators have also shown increased resistance to *H. capsulatum* by prior infection or by immunization. Several routes of inoculation have been used, including intraperitoneal, intravenous and others. The introduction of aerosolized cells of *H. capsulatum* into experimental animals by the airborne route has not previously been investigated. For these reasons guinea pigs were exposed to aerosols containing *H. capsulatum* particles with a view to determining whether they would become histoplasmin sensitive.

The procedures and results can be summarized best as: Laboratory studies demonstrating histoplasmin sensitivity in guinea pigs.

In the preparation of fungal cell suspensions, the Scotchfield isolate of *H. capsulatum*, originally cultured from a human case of histoplasmosis, was used in all of these investigations. Mycelial phase cultures of the organism were grown on the surface of Sabouraud's dextrose agar contained in 250-ml Erlenmeyer flasks at room temperature. The age of the cultures used varied from 3 days to 5 weeks. Fifteen ml of sterile physiological saline solution was introduced into each flask and then sterile glass beads were added. The flasks were rotated to dislodge the mycelium from the surface of the medium and the cell suspensions were transferred to sterile centrifuge tubes. Mycelial particles were washed 3 times by centrifugation and then resuspended in sterile saline. This suspension was transferred to sterile 50-ml vaccine bottles and sterile glass beads added to make up one third of the total volume. The suspension was shaken for 4 hours on a Kahn shaker. Fungal particles were removed with a sterile pipette and a hemocytometer count was made on this suspension. The combination of spores and mycelial fragments was counted and these will be referred to as mycelial particles. The particles were then either killed by addi-

tion of sufficient formalin to make a 1 per cent concentration or diluted to make appropriate viability determinations The former suspensions were cultured for viability after storage in the dark for at least 1 week by transferring several loopfuls to Sabouraud s dextrose agar slants Initial viabilities of live cultures were determined by plating 0 5 ml of 2 concentrations of the suspension onto the surface of Sabouraud s dextrose agar plates These plates were incubated at room temperature and colony counts made at 14 and 21 days

Yeast phase cultures of *H capsulatum* were grown at 37° C on the surface of brain heart infusion agar slants containing 5 per cent blood Growths from 3 to 4 day-old cultures were suspended in sterile physiological saline containing 0 1 per cent cysteine washed 3 times by centrifugation and resuspended in cysteine saline Hemocytometer counts were made on this suspension Cells were then either rendered nonviable by adding formalin to a final concentration of 0 5 per cent or diluted to make appropriate viability determinations Growth determinations were made on the formalin treated suspensions after storage at room temperature in the dark for at least 1 week Sterility was indicated by failure of the organisms to grow on brain heart infusion blood agar at 37° C or room temperature Initial viabilities of live yeast suspensions were determined by plating out 0 5 ml quantities of 2 concentrations onto the surface of Rowley s medium[18] contained in 250-ml Erlenmeyer flasks The flasks were incubated at room temperature and colony counts were made after 12 days

The aerosols used in these experiments were produced in an apparatus originally developed by D W Henderson The techniques used and the formulae for the determination of the spray factors and suspension concentrations were those outlined by Elberg and Henderson[19] The Porton all glass short stem impinger was used as the sampler and the Collison nebulizer as the atomizing instrument The total flow on the machine was 28 l/min the spray flow through the nebulizer 8 l/min The collection or impinger fluid was physiological saline containing 1 0 per cent gelatin to which was added Dow Corning Antifoam Emulsion A Ten drops of Antifoam A were delivered through a 26-gauge needle to 100 ml of the saline-gelatin solution In each study 10 impingers each containing 10 ml of fluid were used to determine the spray factor The aerosol was sampled before and after exposure of the guinea pigs One ml and 0 1 ml samples of liquid from each impinger were plated onto the surface of either Sabouraud s dextrose agar plates or Rowley s medium contained in flasks The former medium was used for counts of viable mycelial particles and the latter for viable yeast cells All plates or flasks were incubated and colonies counted as previously described

Guinea pigs of the Hartley strain weighing optimally 350 to 400 gm were the experimental animals used All animals were skin tested prior to each experiment and at weekly intervals thereafter with 0 1 ml of a 1 10 dilution of histoplasmin HKC 7 Tests were read at 24 and 48 hours Induration of 5 mm or greater was considered a positive reaction at either time interval Control guinea pigs those not exposed to cells of *H capsulatum* were included with each experimental group These animals were skin tested along with the exposed group to rule out the possibility of tissue sensitivity development as a result of frequent exposure of the animals to histoplasmin

Sensitization of Guinea Pigs Using Formalin Inactivated Mycelial Particles and Yeast Cells

Groups of guinea pigs were given a series of injections of nonviable yeast or mycelial phase cells of the fungus This study served as a check on the antigenicity of the cells that were to be used subsequently for attempted airborne induced tissue sensitivity In preliminary investigations, using suspensions of whole cells and cell wall material, it was found that these materials could elicit tissue allergy to histoplasmin when injected subcutaneously into guinea pigs In the present study, guinea pigs were inoculated subcutaneously with 0 2 ml of a nonviable suspension of mycelial particles or yeast cells. In both instances the suspensions contained 2 million cells per ml These injections were administered biweekly until the pigs became positive or the experiment was terminated

TABLE 1

SENSITIZATION OF GUINEA PIGS BY A SINGLE EXPOSURE TO HENDERSON AEROSOL AND BY SUBCUTANEOUS INJECTIONS USING MYCELIAL PARTICLES AND YEAST CELLS OF *HISTOPLASMA CAPSULATUM**

Inoculum	Route of inoculation	No. guinea pigs per group	No. guinea pigs positive to 1 10 histoplasmin	Per cent positive	No. wee required sensitize
Mycelial particles†	Aerosol 600/g 1 μg	13	8	61 5	5 to 10
	Subcutaneous	12	12	100 0	8 to 10
Whole yeast cells‡	Aerosol 3900/g pig	12	8	66 7	4 to 12
	Subcutaneous	12	6	50 0	4 to 18
None (control)		12	0	0	—

* Formalin killed.
† Particles from 3 day-old cultures experiment terminated after 10 weeks.
‡ Experiment terminated after 20 weeks

In the first experiment (TABLE 1) 12 of 12 guinea pigs became sensitive to histoplasmin when nonviable mycelial particles were injected subcutaneously and 6 of 12 guinea pigs became sensitive when nonviable whole yeast cells were used The time required was 8 to 10 weeks for the mycelial group and 4 to 18 weeks for the yeast cell group

In the second experiment (TABLE 2), 10 of 10 guinea pigs became sensitive to histoplasmin when nonviable mycelial particles were used and all of 10 guinea pigs became sensitive when nonviable whole yeast cells were used The number of weeks required was 5 to 12 in the first case and 7 to 10 in the latter

Two groups of guinea pigs were exposed to aerosols of formalin inactivated mycelial particles of *H capsulatum* The first group received a single 1 min exposure to the aerosolized suspension containing 2 million particles per ml The second group of animals was exposed twice to the aerosol the second exposure being 6 weeks after the first The aerosolized suspension in this case contained 1 million particles per ml and the lengths of exposure were 1 and 2 min respectively

Two groups of guinea pigs were exposed to aerosols of formalin inactivated yeast cells of the organism in the same manner as described for inactivated mycelial particles. One group received a single 1 min. exposure to an aerosol nebulized from a suspension containing 2 million yeast cells per ml., and the second was exposed twice to aerosols nebulized from a suspension containing 1 million cells per ml. The lengths of exposure were again 1 and 2 min., with the second exposure given 6 weeks after the first.

With a single exposure to aerosolized nonviable mycelial particles 8 of 13 (61.5 per cent) of the guinea pigs became hypersensitive to histoplasmin in 5 to 10 weeks (TABLE 1). When 14 animals were exposed twice to aerosols of particles, 100 per cent of the animals became sensitized in 7 to 10 weeks (TABLE 2).

A single exposure of guinea pigs to an aerosol of nonviable whole yeast cells of *H. capsulatum* was sufficient to sensitize 8 of 12 (66.7 per cent) in 4 to 12 weeks

TABLE 2

SENSITIZATION OF GUINEA PIGS BY TWO EXPOSURES TO HENDERSON AEROSOL AND BY SUBCUTANEOUS INJECTIONS USING FORMALIN KILLED YEAST AND MYCELIAL PHASE CELLS OF *HISTOPLASMA CAPSULATUM*

Inoculum	Route of inoculation	No. guinea pigs per group	No. guinea pigs positive to 1:10 histoplasmin	Per cent positive	No. weeks required to sensitize
Mycelial particles	Aerosol†	14	14	100	7 to 10
	Subcutaneous	10	10	100	5 to 12
Whole yeast cells	Aerosol‡	10	8	80	7 to 11
	Subcutaneous	10	10	100	7 to 10
None (control)	—	14	0	0	—

* Experiment terminated after 20 weeks.
† Five hundred particles/g. pig initial dose; 1000/g. pig after 6 weeks.
‡ Nineteen hundred cells/g. pig initial dose; 3800/g. pig after 6 weeks.

(TABLE 1). When animals were given 2 exposures to this aerosol, 8 of 10 (80 per cent) converted to a positive skin test (TABLE 2).

Sensitization of Guinea Pigs by Exposure to Aerosols of Viable Mycelial Particles and Yeast Cells

Guinea pigs were exposed to aerosolized viable mycelial particles. According to calculations made from initial viability of cells in the nebulized suspension and from the results of plate counts made from impinger fluid, each guinea pig received approximately 3 viable particles. Eleven of 13 (84.6 per cent) of the animals became sensitive to histoplasmin in 1 to 6 weeks (TABLE 3).

Animals also were exposed to an aerosol of viable yeast cells of the fungus. The calculated number of living yeast cells inhaled per pig in those groups was 76. Ten of 11 (90.9 per cent) became sensitive to histoplasmin within 3 to 4 weeks (TABLE 3).

In calculating the dose of living inoculum, an estimation was made that each guinea pig would inhale either 500 particles or yeast cells of *H. capsulatum*

This was based on our experiences with the viability of the inoculum. However, the inoculum proved extremely fragile during the procedure of atomization. Although the viability of the mycelial phase is quite low, allowance was made for this factor in the preparation of the suspension concentration. This has not been the case with the yeast, in which the viability is usually greater than 30 to 35 per cent. Because of the diverse viabilities of the inoculum it would seem inconceivable that one could state unequivocally that sensitivity was due to the viable particles or cells. However, one significant observation can be made in that the sensitivity occurred much more rapidly in the experiment where small numbers of living particles or yeast cells were present.

TABLE 3

HISTOPLASMIN SENSITIVITY IN GUINEA PIGS AS A RESULT OF EXPOSURE TO AEROSOLS OF VIABLE MYCELIAL AND YEAST CELLS OF *HISTOPLASMA CAPSULATUM*

Inoculum	No. viable cells per guinea pig	No. guinea pigs per group	No. guinea pigs positive to 1:10 histoplasmin	Per cent positive	No. weeks required to sensitize
Mycelial particles	3	13	11	84.6	1 to 6*
Whole yeast cells	76	11	10	90.9	3 to 4†
None (control)	0	13	0	0	—

* Experiment terminated after 14 weeks.
† Experiment terminated after 16 weeks.

Skin Testing of Uninoculated Control Guinea Pigs

In the three experiments a total of 39 uninoculated control animals was given regular weekly histoplasmin skin tests. No conversion to a positive reaction occurred (TABLES 1, 2, and 3). This fact should be emphasized since the test antigen was used in a dilution of 1 to 10. Some investigators would discourage the use of histoplasmin of such potency, although no unequivocal evidence exists that supports that sensitivity results from its use. The control animals in these investigations received a great number of skin test dosages over a period of 16 to 20 weeks. It would suggest that the sensitivity engendered in the experimental animals must have been of a low order.

Studies on Viability of Mycelial Particles of Histoplasma capsulatum

The number of infectious or sensitizing particles must play an important role in the epidemiology of histoplasmosis. If sensitivity to histoplasmin in humans results only from living inoculum, then the percentage of viable cells of an isolate may be the limiting factor. This was not found to be true for experimental guinea pigs, in which nonviable particles stimulated the reactions.

Does skin test sensitivity to histoplasmin signify immunity to [illegible] mosis? One of the goals of the airborne studies was to determine [illegible] ship of histoplasmin sensitivity to immunity. The procedure was [illegible] natural conditions by using the mycelial phase for the challenge [illegible]. Unfortunately, early in these studies viability of the aerosol [illegible] proved to be a serious problem. Impinger samples plated on [illegible]

an extremely low viability and, in some instances, there was no growth from the sample

To determine the degree of immunity elicited in the experimental animals a series of sensitized animals was to be challenged with varying doses of viable inoculum This could not be done with certainty, as demonstrated by preliminary results with aerosols containing live mycelial particles Past experiences with the mycelial phase had shown that the viability of most isolates on artificial media was low However, no thorough study of the viability of mycelial phase suspensions had been conducted In view of these factors it was necessary to make a complete study of the viability of the isolates that were to be used in the airborne investigations

TABLE 4
VIABILITY OF MYCELIAL PARTICLES FROM FOUR ISOLATES OF *HISTOPLASMA CAPSULATUM**

Isolate	Age of culture in weeks													
	0 5		1		2		3		4		5		6	
	Av no colonies per plate†	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability
Scritchfield	9 4	3 1	16 9	5 6	52 8	17 6	3 3	1 1	15 1	5 0	43 0	14 3	49 3	16 4
Santhuff	2 6	0 9	57 8	19 3	2 8	0 9	16 6	5 5	5 8	1 9	33 1	11 0	0 1	0 03
Stimpson	12 1	4 0	48 6	16 2	3 8	1 3	12 4	4 1	17 2	5 7	32 2	10 7	2 8	0 9
Gangel	2 0	0 7	27 4	9 1	0 5	0 2	0 5	0 2	0 5	0 2	0 3	0 1	9 1	3 0

* Three week-old seed cultures
† Ten plates for each determination plates each seeded with 300 particles

The viability of mycelial particles prepared from 4 recent isolates of the fungus was determined These cultures were originally isolated from human cases of histoplasmosis Each isolate was grown on Sabouraud s dextrose agar for 3 weeks at room temperature and designated a seed culture The mycelial particles were harvested and prepared as previously discussed, and a series of 250-ml Erlenmeyer flasks containing 25 ml of media were seeded with each isolate At designated periods flasks were selected randomly and the inoculum harvested for each isolate In each instance approximately 300 particles were plated on each of 10 plates of test medium for viability determinations In this procedure all mycelial components, not only spores were counted In TABLE 4 are presented the average numbers of colonies per plate and the per cent viability obtained from each suspension In FIGURE 1 the data are shown graphically It is evident that none of the 4 isolates had a viability greater than 20 per cent The highest viability occurred within the first 2 weeks of incubation in all 4 isolates In 3 of the isolates there was a precipitous drop in viability during the second week after seeding The Scritchfield isolate had the highest viability at 2 weeks and dropped from 17 6 to 1 1 per cent

viable between the second and third weeks of incubation (TABLE 5) The Gangel isolate had a viability of 9.1 per cent at 1 week then dropped to less than 1.0 per cent and remained low during the next 5 weeks In the other isolate there was a rise in viability at either 4, 5, or 6 weeks There were many

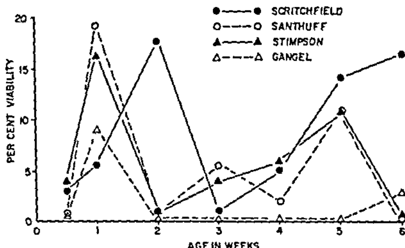


FIGURE 1 Vial dity of mycelial particles from four isolates of *Histoplasma capsulatum* three week-ol l seed cultures)

TABLE 5

EFFECT OF AGE OF SEED CULTURES ON THE VIABILITY OF MYCELIAL PARTICLES OF *HISTOPLASMA CAPSULATUM* (Scritchfiel l Isolate)

Age of seed culture weeks)	Age of culture in weeks: 1		2		3		4		5		6	
	Av no colonies per plate*	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability	Av no colonies per plate	Per cent viability
2	128.9	43.0	31.3	10.4	14.7	4.9	38.3	12.7	24.0	8.0	33.0	11.0
3	16.9	5.6	52.8	17.6	3.3	1.1	15.1	5.0	43.0	14.3	49.3	16.4
6	80.4	26.8	33.2	11.1	30.6	10.2	15.7	5.2	14.1	4.7	21.7	7.2

* Ten plates for each determ nati n plates each seeded with 300 particles

factors perhaps associated with these rises in viability percentages but spore production and germination played a significant role

The findings in these stud es raised the question as to what effect the age of the seed culture may have on the viability of an isolate The Scritchfield isolate was chosen for study because of its uniform growth and because we had prel minary data on its behavior in the Henderson apparatus In

TABLE 2 the average number of colonies and per cent viability by age of culture from 2, 3, and 6-week seed cultures are presented. In FIGURE 2 the comparison of age of seed culture and viability by weeks is shown. It is obvious, with the Scritchfield isolate, that the inoculum from a 2 week-old seed culture has the highest viability after 1-week transfer to new medium. In all of the additional studies no greater than 43 per cent viability was observed.

The viability factor is an important one in inhalation studies when the Henderson apparatus is employed. The suspension of inoculum atomized by the Collison Nebulizer must contain particles of a certain size range. Therefore, it is mandatory that hemacytometer counts be made of the inoculum to

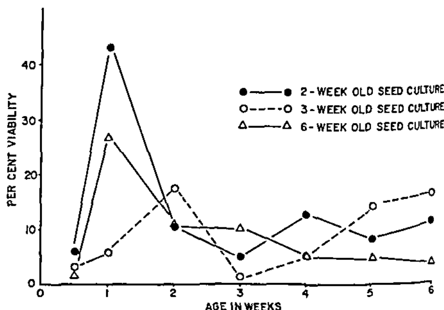


FIGURE 2. Effect of age of seed cultures on the viability of mycelial particles of *Histoplasma capsulatum* (Scritchfield Isolate).

determine the kind of components and the size distribution of the particles in the suspension. Unfortunately, there are disadvantages that have become apparent in our experiences in that mycelial particles, as well as yeast cell seem to be quite fragile. Attempts to increase the efficiency in the atomization of the particles by further breaking up the mycelium led to a much lower viability of the inoculum. A great many problems still need to be solved before a satisfactory challenge with live mycelial particles can be obtained with aerosols in the Henderson apparatus. However, challenge with living yeast cells is quite feasible, but not infallible.

Summary

High histoplasmin sensitivity rates among school children in areas of low endemicity for histoplasmosis have been reported in Georgia and Michigan

A definite reversal in the usual higher incidence of positive skin test reactors in rural as compared to urban residents was established. The epidemiological studies suggested the possibility of a source or sources of inoculum within the urban locations. Direct or indirect contact with these sources resulted in significant histoplasmin sensitivity rates among the urban residents.

Acute histoplasmosis epidemics have occurred in Wisconsin and Missouri; the nature of these permitted thorough epidemiological studies. Illness could be correlated with the activity of the individuals at the sites of each epidemic. *H. capsulatum* was isolated from point sources within the environs of each city. Bird droppings were obvious at the site of the outbreak in Missouri, but none were as evident in the soil samples from the Wisconsin area.

The development of experimental histoplasmin sensitivity in guinea pigs has been investigated. Initial studies showed that tissue sensitivity could be elicited by subcutaneous or intranasal inoculation of either whole yeast cells or yeast cell walls of *H. capsulatum*. In each of these instances a series of inoculations was used. Positive skin reactions were best demonstrated 24 hours after intradermal injection of histoplasmin.

Efforts to develop histoplasmin sensitivity in guinea pigs by exposure to aerosols produced in the Henderson apparatus have proved highly successful. Large numbers of these animals have been sensitized by exposure to aerosols of viable and nonviable mycelial particles of *H. capsulatum*. This has been accomplished in many instances by the use of small inoculum. Challenge of guinea pigs by exposure to aerosols of viable mycelial particles of the pathogen has yielded scanty sporadic results. The low viability of mycelial particles has been a deterrent in this regard and this fact prompted a thorough study of the whole question of viability. It has been found that many factors influence the viability of mycelial particles of the fungus, the most important being age of the culture. One- to two-week-old cultures have consistently yielded the highest viabilities.

Yeast cells of *H. capsulatum* have been found suitable for aerosol studies utilizing the Henderson apparatus. It has been necessary, however, to alter many previously existing experimental procedures in view of the slow growth and fragile nature of these cells.

Acknowledgments

I express my sincere appreciation to Michael L. Furcolow and his staff for their continued support and aid in these studies. The aerosol investigations were done at the Kansas City Field Station, Public Health Service, Kansas City, Kans.

Special gratitude is due my colleague, George C. Cozad, for his assistance in the preparation of the manuscript and for his expert technical assistance throughout these studies.

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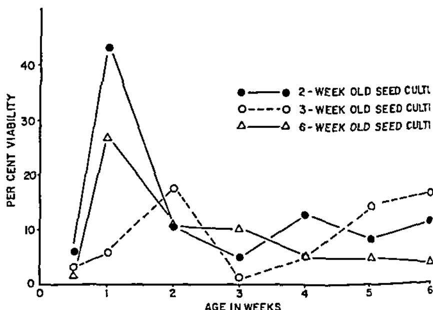


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EMMONSIA CRESCENS SP N AND ADIASPIROMYCOSIS (HAPLOMYCOSIS) IN MAMMALS

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Studies of host range, ecology, and natural history of *Coccidioides immitis* Rixford and Gilchrist, in the deserts of southern Arizona (Emmons 1942, Emmons and Ashburn 1942) revealed that many rodents in that area had mycotic pulmonary lesions Two fungi were isolated in culture from these animals One fungus, *C immitis*, was isolated from 25 of 303 rodents The other, previously unknown, was isolated from 101 of 303 animals distributed among seven species Nine of 303 animals had double infections and both fungi were isolated in culture from these animals

The new fungus was found only in pulmonary tissue where it appeared in the form of spherical cells which reached a diameter of 14μ but did not bud or exhibit any type of reproduction When isolated in culture, the fungus colony grew rather slowly on glucose peptone agar at room temperature, reaching a diameter of 2 inches in 2 weeks (FIGURE 1) Some strains produced white, fluffy colonies, others developed glabrous zones, more or less concentric in arrangement and some strains produced short coremia Conidia were nearly spherical (3 to 4μ in diameter), but with the vertical axis perceptibly shortened so that spores appeared slightly flattened They were borne on simple or compound conidiophores (FIGURE 2) In culture the fungus bore some resemblance to *Histoplasma capsulatum* Darling, but it did not produce macroconidia In tissue its parasitic form was much larger than that of *Histoplasma*, and it did not reproduce by budding

Young cells of the parasitic forms of the two fungi resembled each other, and this made it difficult at first to differentiate them Further examination of pulmonary sections of many naturally infected animals comparisons of the characteristics of fungus cells seen in sections with those of the fungi isolated from the same animal, and experimental infections of laboratory mice (in which the new fungus reached diameters of 35μ) finally resolved this confusion (Ashburn and Emmons, 1945) The new fungus never reproduced in tissue, and it stained more deeply with eosinate methylene blue and induced much less host reaction than did *C immitis* (FIGURES 3 4) In culture there was no resemblance to *C immitis* Nevertheless, the close association of the two fungi in pulmonary mycoses in desert rodents and their similarities during early stages of parasitic growth led to the conclusion that they were genetically related Moreover there were serologic cross reactions that indicated possession of common antigens

Emmons (1942), having accepted the interpretation that *C immitis* is a Phycomycete, compared the new fungus with members of some of the anomalous

genera of that class in an attempt to identify the new fungus Although the hyphae were thinner than those of most Phycomycetes, there appeared to be some resemblance to *Haplosporangium* Thaxter Through the courtesy of David Linder, the fungus was compared with the herbarium material of Thaxter and it was concluded that the fungus might be placed in *Haplosporangium* Accordingly, it was named *Haplosporangium parvum* Emmons and Ashburn the specific epithet referring to its smaller dimensions in culture than those of *Haplosporangium bisporale*

Dowding (1947*a*) reported a fungus similar in culture to *H parvum*, but with spherules reaching a diameter of 270μ, in the lungs of the red squirrel and white-footed mouse in western Canada She isolated the fungus in culture and identified it by its conidial and mycelial characters with the Arizona fungus but noted the greater size of its parasitic form Jellison in 1944 had observed in the lungs of a rock rabbit, *Ochotona princeps*, a parasite that he was unable to identify or to have identified, and he reported this in 1947 as an undetermined parasite (Jellison, 1947) Later he (Jellison, 1950) observed or isolated in culture from 61 Montana mammals a fungus that he recognized as similar to the fungus reported by Dowding From his collections and studies it became apparent that a fungus of this type is frequently present in the lungs of rodents (including the pine squirrel, white footed mouse, and such aquatic rodents as the muskrat and beaver) and in the cottontail rabbit, mink weasel, pine marten and skunk Jellison also demonstrated its widespread geographic distribution in the northern United States, Korea, Japan Africa and Sweden (Jellison, 1950, 1954, 1956, 1958) and in Norway, Finland, France Yugoslavia and Ecuador (Jellison *et al* 1959)

Erickson (1949) reported a similar fungus (without cultures) in the beaver in Minnesota Bakerspigel (1956) found it in the white footed mouse in Saskatchewan McDiarmid and Austwick (1954) found the fungus present in a very high percentage of moles in England, and Tevis (1956) reported he had seen in the lungs of several species of rodents in England objects which in retrospect, he believed were spherules of *Haplosporangium* Because of the isolation of the fungus from animals in so many widely separated geographic areas it is apparent that the original concept of *Haplosporangium* as a desert species with a limited geographic distribution is erroneous

It is reasonable to suppose that the natural habitat of *Haplosporangium* is soil and that this is the source from which mammals are infected Menges and Habermann (1954) have reported the isolation of this fungus from soil in Missouri, but their description of the histopathology of experimentally infected animals arouses doubt as to the correctness of the identification

FIGURE 1 Colony of *Emmonsia parva* on modified Sabouraud's agar after incubation for 2 weeks at 30° C

FIGURE 2 Immature conidiophores and conidia (aleuriospores) of *E parva* ×1120

FIGURE 3 *E parva* and *Coccidioides immitis* in naturally infected pocket mouse (*Perognathus sp*) One small adiaspore of *E parva* is outside the coccidioidal granuloma and two have been incorporated into the granuloma ×250

FIGURE 4 Mature adiaspore of *E parva* in lung of experimentally infected white mouse ×400


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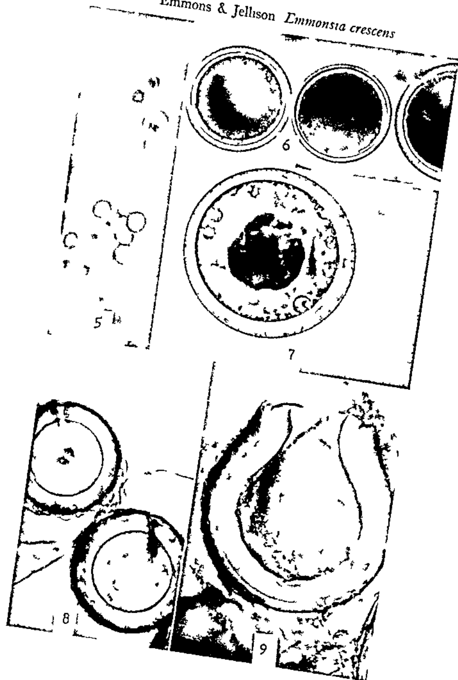
Emmons, after examining their fungus, reported to them that the culture bears some resemblance to *Haplosporangium* but is unlike any strain I have seen" Ciferri and Montemartini (1959) reported that Ciferri isolated *H parvum* from soil in Italy

The systematic position of *H parvum* within the Phycomycetes has been challenged justifiably by several mycologists Continued studies of naturally infected rodents and of experimentally infected mice and guinea pigs fail to reveal any type of sporulation in the large spherical *Coccidioides*like cells of the fungus in its parasitic form in the lung We have long accepted the opinions of other mycologists that the fungus was improperly placed in the Phycomycetes and in the genus *Haplosporangium*

Dowding (1947*b*) discussed the taxonomic position of *H parvum* and called attention to the resemblance of this species to *Histoplasma* and *Blastomyces* Carmichael (1951) reinvestigated the question of segregating the northern North American strains of the fungus (which reach a diameter of 400μ in lung tissue) in a separate species, but he concluded that there were strains intermediate in size and that a clear separation on the basis of differences in dimensions could not be made He stated that the fungus must be excluded from *Haplosporangium* Thaxter and that its closest relationship is to *B dermatitidis* Breslau (1955) stated that the cell wall of *Haplosporangium* is composed of cellulose lying in a protein matrix relatively free of phospholipid while the wall of *Coccidioides* "contains chitin, which lies in a protein matrix having an exterior wall of phospholipid" He stated that the two fungi therefore, were not related Blank (1957), however, found no cellulose in the cell wall of *Haplosporangium*, but demonstrated the presence of chitin

Ciferri and Montemartini (1959) have created a new monotypic genus *Emmonsia*, for *H parvum* We accept this designation, but with some reluctance, because we expect that a suitable earlier generic name eventually may be found It seems improbable that a fungus that so frequently invades the lungs of rodents and presumably inhabits soil or humus should have escaped the notice of mammalogists and mycologists until 1942 Jellison *et al* (unpublished data, 1959) found this fungus in the preserved lungs of a rodent (*Microtus agrestis*) trapped as long ago as 1845 in Sweden The pulmonary lesions in rodents are conspicuous, and the individual fungus cells themselves with a diameter reaching 480μ, are often visible to the unaided eye just below the lung pleura The fungus grows slowly but freely on ordinary culture media at temperatures of 20 to 30° C, and it may have been isolated in the past by soil microbiologists However, a search of mycologic literature has failed to reveal a genus suitable to contain this fungus In culture, in spite of the resemblance of its conidia (aleuriospores) to the microconidia of *Histo*

FIGURE 5 *E crescens* Conidiophores and conidia (aleuriospores) ×1575
FIGURE 6 *E crescens* Adiaspores after 3 weeks incubation at 37° C on blood agar ×205
FIGURE 7 *E crescens* Adiaspore after 8 months incubation at 37° C on blood agar ×205
FIGURE 8 *E crescens* Adiaspore after 8 months incubation at 37° C on blood agar ×205
FIGURE 9 *E crescens* Adiaspore after 8 months incubation at 37° C on blood agar Note irregular fracture of wall and partial loss of contents ×205


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plasma, it never produces macroconidia of the *Histoplasma* type, and its tissue form differs from that of *Histoplasma* by its enormous size and its failure to bud or reproduce in any manner In culture it differs from *B dermatitidis* by the uniformity of size of its conidia and in tissue by its large size and failure to bud

Geomyces vulgaris Traaen, which Ciferri and Montemartini (1959) state "is strictly allied, if not identic [*sic*], with *H parvum*," is distinctly different in growth habit and morphology from *H parvum* We have examined what we believe to be authentic cultures of this fungus obtained from the American Type Culture Collection (No 6685), Washington, D C, and from the Centraal bureau voor Schimmelcultures Baarn, Holland, and we agree with Bisby (1945) that this fungus belongs in the genus *Aleurisma* of Vuillemin The conidiophores of *Aleurisma* are tapered and diverge from the vegetative mycelium at an acute angle and most aleuriospores are asymmetrical, with one side more convex than the other This genus does not produce large spherules in the lungs of experimentally infected animals nor when incubated at 37° C In fact, temperatures as high as 30° C inhibit its growth Other fungi mentioned by Ciferri and Montemartini such as *Glenosporella*, *Botryotrichum*, and *Staphylotrichum*, seem to us to be so different from *H parvum* in colony and morphologic characteristics that transfer to one of these genera need not be considered We therefore follow these mycologists and accept the name *Emmonsia parva*

In our experience mature cells of *Emmonsia* in pulmonary tissue of naturally or experimentally infected animals fall into two groups, usually well separated on the basis of size This obvious difference is correlated with other differences that are more fundamental in specific differentiation Accumulated experience with several hundred isolates of these fungi including experimental infections with many strains, induces us to describe the large form as a new species The specific name refers to the parasitic form of the species and signifies its increase in size

Emmonsia crescens n sp Colony white, reaching a diameter of 3 to 5 cm in 10 days at 30° C on Sabouraud's agar Growth poor on cornmeal and Czapek's agar media Colony varying with the strain from floccose and uniform throughout to coremiform or with irregularly scattered or zonate glabrous areas, mycelium delicate, hyphae 0 5 to 2μ in diameter, septate freely branching, conidiophores arise at right angles from vegetative hyphae varying from simple, short lateral branches 1 to 2 × 2 to 10μ to compound and 2 to 5 × 8 to 20μ, with secondary short conidiophores arising at right angles from the primary conidiophores, conidia (aleuriospores) borne single or rarely in chains of two, subspherical, slightly shortened along the vertical axis, 2 to 4 × 2 5 to 4 5μ, minutely spiny, adherent to the conidiophore (FIGURE 5)

FIGURE 10 *E crescens* Adiaspore in lung of *Arvicola terrestris* Natural infection type specimen ×63

FIGURE 11 Same as #10 Note minimal host reaction ×63

FIGURE 12 *E parva* Adiaspores after incubation for 3 weeks at 40° C on blood agar ×400

FIGURE 13 *E parva* Adiaspore from experimental infection in white mouse Note single nucleus ×1575

When incubated at 37° C on any media, but preferably on an enriched medium such as glucose blood agar, the conidia and some hyphal cells enlarge to spherical multinucleate cells up to 400μ in diameter with walls reaching a thickness of 70μ, no budding endosporulation or reproduction of any type observed, although metabolic products and eventually degeneration artefacts may superficially resemble endospores (FIGURES 6–9)

These large cells appear to be identical in nature to the parasitic form of the fungus seen in lungs of animals (FIGURES 10, 11) In animal tissue, inhaled or experimentally introduced conidia enlarge to multinucleate spherical cell that may reach a diameter of 480μ, walls strongly Schiff positive, up to 70μ thick of two definite layers, the outer surface rough or sculptured, mature cells contain many nuclei 1 to 1 5μ in diameter and small globules of metabolic materials that usually coalesce to form one or two irregularly shaped bodies no budding, endosporulation or reproduction of any kind, calcium may be deposited in the fungus cell after its death Presence in pulmonary tissue especially if fungi are few, induces minimal host tissue response during early stages of growth Fungi, when present in very large numbers, reduce effective lung tissue by mechanical displacement Recovery in culture is easily accomplished with immediate reversion to mycelial form if cultured at 20° to 30° C

The habitat is presumably soil, humus, or decomposed vegetation, but known principally from the lungs of several species of rodents and other animals

We designate as the type culture an isolate from the lungs of a rodent *Arvicola terrestris*, captured at Hamar, Norway, in 1959 Sections of tissue and cultures have been deposited in the American Type Culture Collection under the number 13704 Sections of the lung of this rodent host are deposited also in the National Mycological Herbarium and in the files of the Armed Forces Institute of Pathology, both in Washington, D C

Emmonsia crescens sp n Coloniis albis et floccosis cultivatis in gelosa cum dextroso peptonoque, hyphis 0 5–2μ diam , conidiophoris ad angulos rectos vel simplicibus et 1–2 × 2 10μ vel compositis et 2–5 × 8 20μ, conidiis cum spinis minutis, breviatis in axe verticali, 2–4 × 2 5–4 5

In pulmonibus animalis et ad 37° C in vitro, conidia possunt fieri cellulas globosas, interdum 480μ diam et cum parietibus 10 70μ cr *

We propose the epithet *crescens* to indicate the enormous increase in size of conidia in animal tissue and *in vitro* at 37° C

We propose the term *adiaspore* for the large spherule which, in the species *E crescens*, grows to a volume 1,000,000 times that of the inhaled or incubated conidium from which it arises

We propose the name *adiaspiromycosis* to designate the pulmonary disease caused by species of *Emmonsia* Adiaspiromycosis is derived from α-δια σπειρειειν-μυκ(ης) osis, a term descriptive of this mycosis in which there is no multiplication or dissemination of the fungus beyond its original site of implantation

* Colonies on dextrose peptone agar white floccose hyphae 0 5–2μ in diameter conidiophores borne at right angles simple and 1 2 × 2 10μ or branched and 2 5 × 8 20μ conidia with minute spines shortened in vertical axis 2–4 × 2 5–4 5μ

In lungs of animals and *in vitro* at 37° C conidia increase in size reaching a diameter of 480μ and with walls 10–70μ thick





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FIGURE 14 *E crescens* Edge of adiaspore from experimental infection in white mouse Note very numerous peripheral minute nuclei ×1575

FIGURE 15 *E crescens* Adiaspores which were incubated for 3 weeks at 37° C on blood agar then removed to room temperature Note multiple germ tubes from two adiaspores and the rupture of one which was not viable ×205

Colonies of *E. crescens* are indistinguishable from those of *E. parva* in culture at 30° C. They fall within the limits of colony variability of that species. *E. crescens* resembles *E. parva* also in morphology, but produces slightly more compound conidiophores. The conidia of the two species are indistinguishable (FIGURES 2-5). However, when incubated on blood agar at 37° C, the conidia of *E. crescens* grow by spherical enlargement from their original size of 2.5 to 4μ to a diameter of 250 to 400μ (FIGURES 6-9). The wall, which is Schiff positive, may reach a thickness of 70μ. *E. parva*, on the contrary, grows in mycelial form at 37° C, but if the temperature of incubation is raised to 40° C some strains of this species produce adiaspores (FIGURE 12) comparable to those of its parasitic form. These cells *in vitro* usually reach a diameter of only 10 to 25μ, with walls not exceeding 2μ thick. In the mouse lung they reach a diameter of 40μ, with a wall 4μ in thickness (FIGURE 13). *E. parva* may be described as more thermophilic than *E. crescens* (which does not grow at 40° C), an attribute perhaps related to its occurrence in hot climates.

The adiaspores of *E. crescens in vitro* and *in vivo* are multinucleate (FIGURE 14), while those of *E. parva* are uninucleate (FIGURE 13) even after reaching their mature size (Ashburn and Emmons, 1945). Adiaspores of both species contain globules of metabolic products that at certain stages may resemble endospores, but usually in older adiaspores these bodies coalesce into one mass. Natural infection has been observed only in the lungs.

When adiaspores of *E. crescens* incubated at 37° C for 2 or 3 weeks are incubated subsequently at 30° C or room temperature, they germinate by producing many hyphae (FIGURE 15). This germination by multiple germ tubes presumably is related to the multinucleate condition of the adiaspore of *E. crescens*. When the incubation temperature of an adiaspore of *E. parva* is lowered from 40° to 30° C, the spore germinates by a single germ tube.

The persistent uninucleate condition of adiaspores of *E. parva* and the multinucleate condition of those of *E. crescens* constitute a remarkable and important difference between these two species. The nuclei of these fungi can be demonstrated only after special handling. The tissues illustrated in FIGURES E and D in the paper by Ashburn and Emmons (1945) and those illustrated in FIGURES 4, 13, and 14 of this paper were processed in the laboratory of one of us (C. W. E.) following procedures outlined in a recent description of fungus nuclei (Emmons, 1959).

There appears to be a geographic separation of the two species, although this must be studied further by additional collections of specimens. In so far as we know, *E. parva* has been isolated in culture only in the arid Southwest, the most northerly collection being from St. George, Utah. One collection from Africa (without culture) may be *E. parva*. *E. crescens*, on the other hand, has been reported or found by one of us (W. L. J.) in Montana, Korea, Sweden, Africa, Norway, Finland, France, Yugoslavia, Ecuador, and, as previously stated, by other mycologists in Canada, England, and Italy.

Summary

Transfer of *Haplosporangium parvum* to Ciferri's and Montemartini's new genus *Emmonsia* as *Emmonsia parva* is noted. *Emmonsia crescens* sp. n. is segregated from the former species on the basis of its greater size in the

adiaspore or parasitic form (200 to 480μ versus 14 to 60μ), a thicker wall (10 to 70μ versus 2μ) the lower temperature required to produce adiaspores *in vitro* (37° C versus 40° C), and the multinucleate condition of the adiaspore *E parva* is known at the present time only from animals in the arid Southwest of the United States and possibly Africa, whereas *E crescens* has been found in animals in the northern United States Canada, England, Korea, Sweden, Norway Japan, Africa, Finland, France Yugoslavia, and Ecuador *E crescens* has been isolated in culture from animals in Canada, the United States, England Norway, Sweden, and Finland An isolate from *Arvicola terrestris* collected at Hamar, Norway, has been designated as the type culture of this new species The pulmonary disease caused by species of *Emmonsia* is designated adiaspiromycosis to signify the absence of multiplication and dissemination of the fungus in the animal host

Acknowledgment

We are indebted to W H Everhardy for preparing the Latin diagnosis of the fungus and devising the name of the disease

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STUDIES OF THE GROWTH AND METABOLISM OF *COCCIDIOIDES IMMITIS*

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The *in vitro* growth of the spherule form of the dimorphic fungus *Coccidioides immitis* has been reported frequently since it was first observed by MacNeal and Taylor[1] in 1914 The literature concerned with the occurrence of the spherules in culture has recently been reviewed by Friese[2] For the most part the early reports described the formation of spherules accompanied by considerable mycelial growth More recently Lubarsky and Plunkett[3] cultured spherules in the absence of mycelium in a medium containing chicken serum and embryo extract under increased carbon dioxide tension An important contribution is that of Converse[4] who devised a simple defined medium and procedure for the conversion of arthrospores and mycelial fragments of strain M 11 *of the fungus to spherules The spherule form was maintained* successfully for three serial transfers

It is the purpose of this report to describe our experience with prolonged cultivation by serial transfer of the spherule form of strain M 11 of *C immitis* and to describe initial experiments intended to contribute to our meager knowledge of the metabolism of this fungus

Arthrospores and mycelial fragments were prepared in shake cultures in a modification of the defined medium of Roessler and his co-workers[5] containing K_2HPO_4 0 015 *M* KH_2PO_4 0 015 *M*, $MgSO_4$ 0 0008 *M* ammonium acetate 0 08 *M* glucose 2 per cent and zinc 2 ppm The magnesium concentration was reduced from the level recommended by Roessler to avoid precipitation of magnesium phosphate

The medium and method of Converse[4] were used for the conversion of arthrospores and mycelial fragments to spherules This medium is similar to that of Roessler but contains the ingredients in a lower concentration and in addition 0 05 per cent of the dispersing agent Tamol N * Ten to 25 ml quantities of the autoclaved medium in Erlenmeyer flasks of a capacity 5 times the volume of medium were heavily inoculated with a suspension of arthrospores and mycelial fragments The initial incubation was for *72 hours at 36° C on a rotary shaker of 1 inch amplitude* and 120 cycles/min At the end of this period many spherules were present some of them mature along with developing mycelium Most of the mycelium could be removed as Converse has pointed out by filtration through several layers of sterile surgical gauze The spherules were harvested by centrifugation and the supernatant fluid discarded The cells were returned to fresh medium and reincubated Thereafter transfers of the filtered centrifuged cultures were at daily intervals with the entire yield of cells returned to the culture until the density of the inoculum amounted to about 1 mg dry weight of cells per

* Rohm & Haas Company New York N Y described as a neutral sodium salt of a condensed aryl sulfonic acid

ml The volume of the culture was then gradually increased at each transfer but never to more than double the previous volume When a culture volume of 100 ml was achieved the density at inoculation was controlled at 2 0 mg of cells per milliliter and the incubation period at 16 hours For continued growth 200-ml cultures in liter flasks were found convenient When larger quantities of cells were wanted multiple cultures were prepared

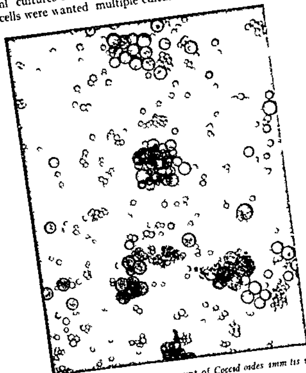


FIGURE 1 Photomicrograph of a wet mount of *Coccidioides immitis* spherules after 41 transfers in liquid culture ×212

The determination of cell densities for the control of size of inocula and the determination of yields of cells were made by filtration of a measured volume of a suspension of cells through a dried and weighed HA Mill pore membrane followed by washing drying to constant weight at 105° C and reweighing Under the conditions of culture here employed the cell density increased from 2 0 mg /ml at the time of inoculation to 4 0 to 4 5 mg /ml at the time of harvest

The development of small amounts of mycelium along with the spherules continued for a time but with repeated transfer the amount of mycelium diminished and the frequency of filtration through gauze was reduced After about 45 transfers the filtrations were discontinued entirely The amount of mycelium present in these well established cultures was very small Although

there is no known method for accurately measuring the proportion of a culture that is in the mycelial form it is felt that the amount of mycelium was certainly less than 1 per cent and probably less than 0 1 per cent of the total cellular material Spherule cultures have been carried in the manner described for as many as 160 consecutive transfers FIGURE 1 is a photomicrograph of a wet mount of such a culture at the forty first transfer

We have recently presented evidence showing that the beneficial effect of a large inoculum on the maintenance of the spherule growth form is due to the greater concentration of metabolic carbon dioxide in such cultures[6]

The availability of an adequate and continuing supply of spherules facilitates the examination of this modification of the fungus in a manner not heretofore possible The remainder of this report will describe the beginning of a study of the metabolic character of this microorganism

Spherules grown as previously described were harvested and washed 3 times with sterile water in the centrifuge Starved or depleted cells were obtained by shaking washed cells at a density of 20 mg /ml in sterile water for 16 hours at 36° C Respiration and fermentation studies were carried out at 37° C in the conventional manner in a Warburg apparatus with 20-ml double side arm vessels Unless otherwise noted flasks contained 1 5 ml of 0 05 *M* phosphate citrate buffer at *p*H 6 0 and 5 0 mg dry weight of cells in the main compartment Either 0 5 ml of water or 20 μmole of glucose in water was placed in 1 side arm and tipped in after equilibration Where needed 0 5 ml of 0 1 N H_2SO_4 was added to the second side arm to release bound carbon dioxide at the end of the experiment Where oxygen uptake was measured 20 per cent KOH solution was present in the center well The total liquid volume was 3 ml Anaerobiosis was created in vessels by sweeping with nitrogen freed of oxygen by passage over hot copper When the contents of vessels were to be analyzed the vessels were chilled in ice water immediately after the final manometer reading opened and the contents quickly filtered through a millipore membrane Glucose determinations were made on the filtrate as reducing sugar by the method of Folin and Malmros as described by Umbreit *et al*[7] Ethanol was determined by the microdiffusion method of Conway[8] Carbon 14 counts were determined with a Packard Tri Carb liquid scintillation spectrometer in methanol toluene water solution with 2,5-diphenyloxazole and *p* bis 2 (5 phenyloxazolyl) benzene as phosphors

In TABLE 1 are summarized the results of a typical experiment in which the rates of respiration and fermentation of spherules were measured over a 3 hour period For comparison the results of a similar experiment performed with the mycelial phase of strain M 11 are included The mycelium was grown in shake culture for 16 hours at 36° C in the modified medium of Roessler previously described The inoculum of 1 mg /ml was less than that of the spherule cultures because of the greater viscosity of the mycelial suspensions At harvest the thick suspension obtained by centrifugation was dispersed by treatment for 30 sec in a chilled blender to facilitate handling of the suspension with pipettes It was washed 4 times with sterile water before use in the metabolic studies The starvation procedure was the same as that described for the spherules but at a density of 10 mg /ml

Washed spherules exhibited an appreciable endogenous respiration that was reduced slightly by depletion Respiration was stimulated by the addition of an excess of glucose Anaerobically fermentation occurred that responded to added substrate It is of interest that starvation of the spherules resulted in a substantial stimulation of the ability of the cells to ferment glucose The reason for the activation of an anaerobic process by exposure of the cells to aerobic conditions in the starvation procedure is not known The same stimulation was not obtained when the cells were shaken for 16 hours in nitrogen Although the spherules possess the capacity for anaerobic metabolism we have been unable to detect any growth of either spherules or mycelium under completely anaerobic conditions

The washed mycelium exhibited a higher endogenous oxygen uptake than the spherules and the rate was affected only slightly by glucose However, the endogenous rate was reduced substantially in the starved mycelium and a response to glucose was now more apparent A notable difference from the spherules is seen in the much lower fermentative activity of the mycelium It would be hazardous to attribute the differences noted solely to the morphologic modifications of the organism since the mycelium was grown in a higher concentration of nutrients and at a lower cell density than the spherules However mycelium grown in the spherule medium from which Tamol *N* was omitted exhibited metabolic characteristics similar to those just described In the mycelium grown on this dilute medium there appeared to be an increased tendency for fragmentation and arthrospore formation which became especially pronounced during the starvation procedure

TABLE 1

EFFECT OF STARVATION AND ADDED GLUCOSE ON RESPIRATION AND FERMENTATION OF *COCCIDIOIDES IMMITIS*

Cells	Added substrate	µL/mg of cells per hour			
		Spherules		Mycelium	
		O_2	Anaerobic CO_2	O_2	Anaerobic CO_2
Unstarved	None	5 6	3 3	13 8	6
Unstarved	20 µM glucose	18 7	7 7	15 7	5
Starved	None	4 5	2 2	4 5	8
Starved	20 µM glucose	18 9	12 6	12 0	1 3

Under anaerobic conditions equimolar amounts of carbon dioxide and ethanol were formed by spherules as seen in TABLE 2 The glucose utilized is in good agreement with the theoretical amount required to account for the fermentation products only if it is assumed that the endogenous fermentation is suppressed by added glucose Alternatively a small amount of glucose may be assimilated

The optimal *p*H for both respiration and fermentation of spherules in buffer 0 03 *M* with respect to citrate phosphate and tris(hydroxymethyl)amino

TABLE 2
FERMENTATION OF GLUCOSE BY COCCIDIOIDES IMMITIS SPHERULES

Added substrate	μM per vessel		
	CO_2	Ethanol	Glucose disappearance
None	1.9	1.9	
20 μM glucose	5.3	5.4	2.7

methane (Tris) was found to lie at or near 6, as seen in FIGURE 2. Initial *p*H values were used in preparing the figure. Measurements at the end of the experiment indicated no change greater than 0.2 of a *p*H unit except at *p*H 9 which dropped to 8.65. Phthalate and borate as buffers were found to inhibit metabolism. Acetate and succinate are oxidized by spherules. In

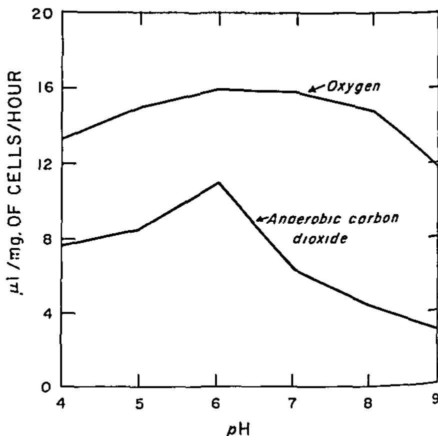


FIGURE 2. Effect of *p*H on fermentation and respiration of *Coccidioides immitis* spherules.

addition at pH 4, acetate was an effective inhibitor of both respiration and fermentation to such an extent that a 0 03 *M* concentration completely abolished the utilization of glucose

In order to determine the amount of glucose assimilated during respiration and the possible occurrence of metabolic products other than carbon dioxide, washed spherules were allowed to respire in the presence of glucose uniformly labeled with carbon 14 Analyses were made at the beginning and after 3 hours of respiration The results are summarized in TABLE 3 It will be seen that approximately one half of the 19 9 μmole of glucose added per vessel was utilized Of this, 14 per cent was converted to carbon dioxide and 15 per cent to as yet unidentified substances remaining in the filtrate It is

TABLE 3

OXIDATION OF C[14] GLUCOSE BY COCCIDIOIDES IMMITIS SPHERULES

Time (hours)	μM O_2 consumed	μM glucose found	Counts per vessel per minute* Filtrate	Counts per vessel per minute* CO_2
0		19 9	20 460	
3	10 0	10 4	12 036	1199

	μM
O_2 consumption in absence of glucose	2 8
Stimulation of O_2 consumption by glucose	7 2
Glucose utilized	9 5
Glucose converted to C compounds in filtrate	1 5
Glucose derived CO_2	8 1
Glucose assimilated (by difference)	6 7

* Background counts have been deducted

hoped that identification of these metabolic products may offer a clue to metabolic pathways in this fungus By difference it may be calculated that 70 per cent of the glucose utilized was assimilated by the cells

In conclusion the ease of prolonged cultivation of strain M 11 of *C immitis* in the spherule phase suggests its use for the evaluation of antifungal agents of potential usefulness in the treatment of coccidioidomycosis In addition the availability of almost unlimited quantities of cells produced in liquid culture recommends the method for production of material for fractionation and immunological studies

An examination of certain of the metabolic properties of washed spherules has shown that respiration is increased by the addition of a metabolizable substrate such as glucose and that most of the glucose utilized is assimilated Under the conditions of these experiments marked stimulation of respiration of the mycelial form by added glucose was obtained only after starvation of the cells *C immitis* is capable of anaerobic metabolism with the formation of carbon dioxide and ethanol from glucose This activity, which is stimulated by starvation is present in a much lower degree in mycelium than in spherules

The optimal *p*H in citrate phosphate Tris buffer for both respiration and fermentation is at or near 6

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APPROACHES TO THE PHYSIOLOGY OF *COCCIDIOIDES IMMITIS**

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The report of Lones and Peacock in this publication provides striking and much needed advances in our knowledge of the physiology of *Coccidioides immitis* It is indeed noteworthy that ethanol is produced by the spherule form This may provide taxonomists with another point of similarity between *Coccidioides* and the Phycomycetes for, as Louis Pasteur showed, *Mucor racemosus* carried out alcoholic fermentation associated with reduced oxygen tension and a morphologic change The significance of this mode of metabolism in pathogenesis by *C immitis* and its possible susceptibility to potential therapeutic agents, for example, tetraethylthiuram disulfide, pose still further questions concerning this pathogen

It is to be expected that further studies of interest and importance will result from the development of spherule culture method by Converse[1] and by their extension by Lones and Peacock and by Cobb and Levine[2] at the Naval Biological Laboratory For example, in addition to metabolic studies, immunological explorations can be carried out on spherules The development of immunity to coccidioidomycosis is supported strongly by clinical as well as by experimental evidence in this respect Various cellular as well as cell free extracts of both spherules and mycelial forms now can be prepared, and improvements can be anticipated in experimental immunization procedures In addition, the vexing problem of achieving success *in vitro* with experimental drugs and then meeting with failure when these are tested against the disease in experimental animals is familiar to all who have worked on the chemotherapy of coccidioidomycosis Moreover spherules *in vitro* may provide a screening method

Preoccupation with the production of spherules should not cause the problem of discouraging spherule formation *in vivo* to be overlooked Two observations relative to this are the following

First, the early stages after inoculation of *C immitis* are attended by an active inflammatory response This is exemplified in FIGURE 1, which shows the vigorous exudation of polymorphonuclear cells even phagocytosis of arthrospores, in alveoli of a mouse inoculated intranasally Spherule formation with progressive disease then would follow if a virulent strain were used

Second, it is recognized that a person with systemic coccidioidal disease but without dermal lesions may occasionally develop suppurative lesions draining at the skin if some trauma of the skin is incurred This may suggest a predilection on the part of the *Coccidioides* cells for the resulting inflamed site These

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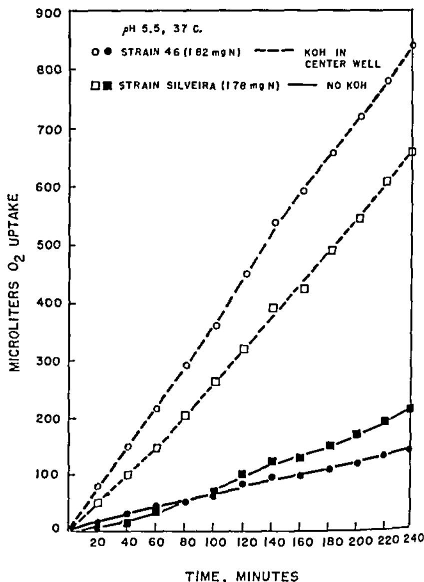


FIGURE 2 Endogenous respiration by mycelia of two strains of *C immitis*

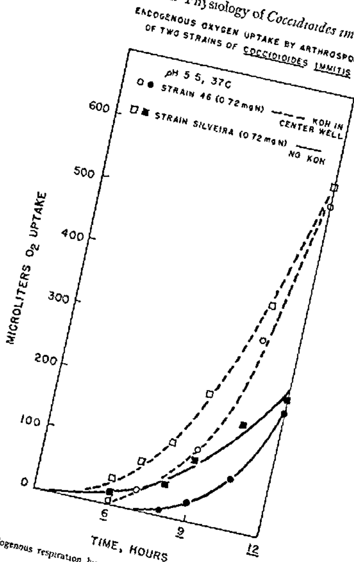


Figure 3 Endogenous respiration by arthrospores of two strains of *C immitis*

Jevoted attention to this in *C immitis* and it is possibly in the area of en logenous physiology that biochemically vulnerable patterns may be sought

The extraction of mycelium with hot ethanol yielded approximately 10 er cent of the dry weight as ether soluble lipid This was a soft yellowish ax that melted at 36 to 38° C Thus far attempts to determine whether this

lipid might constitute the endogenous substrate, compatible with an RQ in the range of 0.7 to 0.8 have given indefinite results. In addition one would wonder about its immunological importance.

Another cellular constituent of *C. immitis* has received the attention of several workers. Baker *et al.*[10] suggested the presence of chitin in the cell wall, and Blank and Burke[11] and Tarbet and Breslau[12, 13] have applied X-ray diffraction and chemical and histochemical methods to determine its presence. We have demonstrated the presence of glucosamine after hydrolysis of cell wall preparations from mycelia by paper chromatography, and by measurement of the optical rotation of the chromatographically isolated material. The $[\alpha]_D^{25}$ for hexosamine isolated from two strains of *C. immitis* were +68° and +71.5° and for known glucosamine, +68°. The values given in *Organic Syntheses*[14] were from +69.8° to +70.1°. Estimation of the hexosamine in the cell wall material by the colorimetric method of Dische and Borenfreund[15] suggested the presence of about 3 to 4 per cent chitin of the dry mycelium weight. Further indication that chitin was present was the liberation of reducing sugar by chitinase isolated from a *Streptomyces* species by D. M. Reynolds.[16]

Tarbet and Breslau[12, 13] have shown chitin in spherules, which may imply a relative resistance of the structure of *C. immitis* to known mammalian enzymes, although of course chitin is likewise recognized in nonpathogenic fungi and per se would not determine pathogenicity.

From time to time many authors have noted the shaggy or prickly appearance of spherules in infected tissue (P. Negroni, personal communication).[17] In FIGURE 4a is shown such a spherule in a section stained with hematoxylin and eosin. The protrusions are visible at the surface and on the endospores within. The same slide was destained and then the periodic acid Schiff stain was applied. As shown in FIGURE 4b, the shaggy border was not polysaccharide; indeed, the spherule wall appears deficient in polysaccharide which is limited to a thin inner ring and possibly a scattering at the periphery of the wall. Of course, one should certainly examine the illustrations of Breslau and Tarbet[12, 13] for a clear notion of the histochemical appearance of *C. immitis*.

However, the periodic acid Schiff stained spherule contains striking endospores with what appears to be a carbohydrate shell as well as polysaccharide intracellularly. Thus far we have not distinguished the types of polysaccharide that may be represented in these two loci. Immunologically, both regions may be significant; for example, if the polysaccharide of the endospore wall can react with precipitating antibody, then agglutination may occur. We have indications that this can take place, although earlier workers using mycelial phase organisms did not observe such a reaction. Perhaps some type of neutralization reaction may be sought in the resistant host or in one with precipitins.

The events leading to the release of immunologically or serologically active materials may be pertinent to the examination of these cellular components. As yet we have not carried out such studies on the *in vitro* spherules, but earlier we described the method of producing antigens by autolysis of young mycelia of *C. immitis*.[18] As is generally known, there are two important

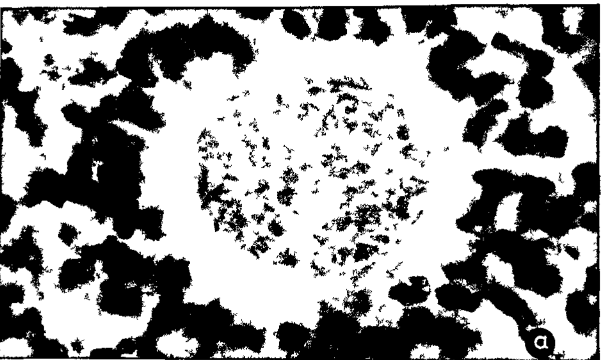


FIGURE 4 (a Sphe u e of *C n mil* n sub u aneous absce s sho ng p kly su face and endo pores Hematoxyl n and eos n

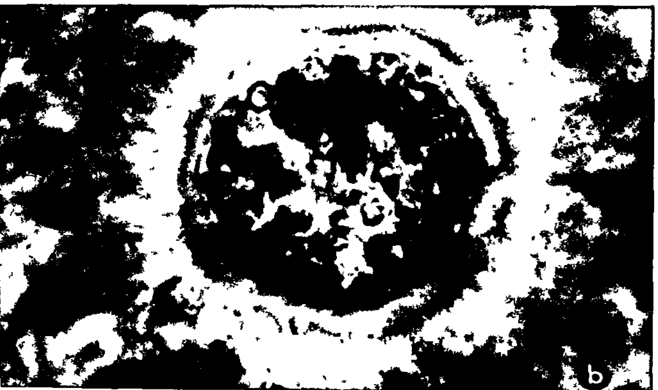


FIGURE 4 (b) Same spherule after destaining and application of periodic acid Schiff stain for polysaccharide. Polysaccharide sparse in spherule wall, conspicuous in wall and inside of endospores.

serologic tests applied in coccidioidomycosis[19] The precipitin test is a diagnostic aid, precipitins appearing early in the course of the disease and usually diminishing early The complement fixation test provides a prognostic criterion of the infection, it becomes positive later and the complement fixing titer rises with worsening of the disease By autolysis of mycelium, antigen solutions were obtained that gave reactions in both precipitin and complement fixation reactions It was not known whether the same or different antigens were involved in the two serologic reactions An approach to this was made

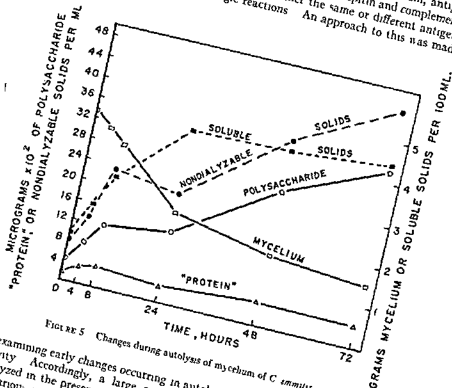


FIGURE 5 Changes during autolysis of mycelium of *C immitis*

by examining early changes occurring in autolysis and by testing for serolo activity Accordingly, a large mass of young (3-day-old) mycelium w autolyzed in the presence of toluene, and aliquots were removed at interva for various assays The measurements obtained are depicted in FIGURE 5 Mycelium weight decreased steadily to about 50 per cent in the first 24 hours, then slowed over the next two days to a final 35 per cent of the original weight Of course, the soluble solids liberated in this period showed a reciprocal rise with the drop in mycelial weight

The nondialyzable solids included the polysaccharide and nitrogenous material designated as "protein" (this reservation is prompted by the fact that this fraction contains amino acids, but its protein nature has not been established)[20] After an initial increase in nondialyzable substances, between

8 and 24 hours there occurred a drop in this fraction The reason for this is not clear but the concomitant drop in nondialyzable protein suggested that the latter material might have undergone lysis which produced smaller constituents removed by dialysis Polysaccharide was continuously liberated although its rate of release was also affected during the 8 to 24 hour period

The serologic activity of the filtrates at different stages was measured against human coccidioidomycotic serum The complement fixation titers of the filtrates (which were totally devoid of anticomplementary activity) were as follows At zero (0) time there was 4+ fixation of complement (2 units) at a dilution of 1 64 An increase in titer was such that 4+ fixation occurred at a dilution of 1 128 of the 4 hour sample The 8 hour and 24 hour specimens gave complete fixation (4+) at 1 256 The titer then rose again and was 1 512 at 48 hours and 1 1024 (the highest dilution tested) at 72 hours

The immediate temptation was to point to the polysaccharide curve of the graph and to suggest that the rise in complement fixing titer was causally associated However we feel that the filtrates must be examined more closely for example for qualitative changes, before such a relationship can be upheld

The precipitin antigen activity of the filtrates was apparently increased during the progressive liberation of nondialyzable substances However our experience with quantitative precipitin tests with *Coccidioides* filtrates indicates that antisera may give precipitates with polysaccharide components and with noncarbohydrate substances accompanying the former Thus mixed precipitating antigens may be present in the autolysates indicated in FIGURE 5

As we have established earlier, the soluble polysaccharide in *Coccidioides* lysates and coccidioidins contains predominantly mannose with some galactose and another reducing sugar [9,10] which Goldschmidt has characterized as monomethylmannose [1] It is of interest that Salvin recently reported mannose and galactose present in serologically active extracts of yeast phase *Histoplasma capsulatum* [27] Perhaps herein lies the basis of immunological cross reactivity of *C immitis* and *H capsulatum*

Although it may appear even more remote from the physiology of *C immitis* one other aspect of work with this fungus deserves mention As we are aware the surroundings may have a pronounced effect on the metabolic activity of an organism For this reason one might expect different behavior of *Coccidioides* by varying its *in vivo* ecology For example as shown in FIGURE 6 a relatively avirulent strain of *C immitis* (strain 46) when given in doses up to 10 000 spores intraperitoneally, had no apparent effect on the health of mice However when given intranasally, as low a dose as 100 cells induced lethal progressive infections Intramuscularly and subcutaneously, as many as 1000 cells did not cause progressive disease The other strain (Silveira) included in FIGURE 6 was highly virulent when given intranasally as well as intraperitoneally By intramuscular route it manifested somewhat less virulence and when given subcutaneously, was localized in a small abscess [25] As recorded in human cases by Wilson *et al* [33] and Trimble and *Doucette* [4] infection with *C immitis* in the skin was arrested before inducing extensive lesions As indicated above for the mouse and by several workers for other animals including the dog sheep calf and guinea pig (K Maddy, personal

communication) [4, 25] subcutaneous inoculation generally resulted in an arrested lesion When 100 spores of strain Silveira were injected subcutaneously into cynomolgous monkeys (*Macacus irus*) the result was a large fluctuant abscess 2 to 3 cm in diameter but in the mouse this dose gave a lesion usually 3 mm in diameter Thus the size of the animal in this case was not significant Intratracheal inoculation of dogs by Hugenholtz *et al* [26] produced severe disease at doses of 1000 spores or more Lowe *et al* [27] and we have similarly induced extensive pulmonary lesions in the monkey by inhalatory exposure to

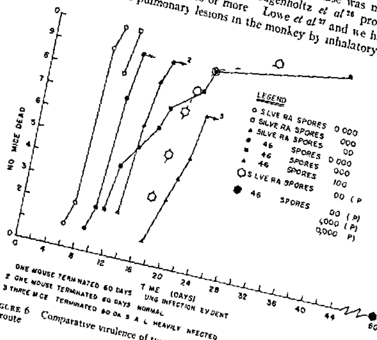


FIGURE 6 Comparative virulence of two strains of *C im nit s* (S lveira and 46) by intra nasal route

arthrospores (we used a dose of 750 spores per monkey) On the other hand Maddy instilled 1 million spores intratracheally in a cow without inducing illness (K Maddy personal communication) The interesting results of Lubarsky and Plunkett showed that *C immitis* could survive passage through the alimentary canal of mice without causing overt disease [28]

On the other hand intravenous inoculation whether carried out in the mouse as by Emmons and Liggott [29] and by L E Gordon (personal communication) in the calf by Giltner [4] or in the horse by C E Smith (personal communication) results in overwhelming infection

These examples raise the question as to the nature of the variation in pathogenesis by different routes of inoculation The lung appears to be a favorable site for development of *C immitis* Is this due to the copious blood supply or the oxygen tension? Is there sufficient difference in the quantity of con-

nective tissue elements between the lungs and subcutaneous region to result in the difference in susceptibility observed in these two tissues? Is there some demonstrable physiological difference, for example, catecholamine-type precursors of melanin, to account for the greater susceptibility to coccidioidal disease in dark skinned persons?[19] Such questions are not unique to medical mycology, but the fungus diseases may permit some direct approaches

Some practicable concepts may derive from such animal studies also First despite continued recommendation or use of guinea pigs by some textbooks or laboratories, it is probable that most investigators who have worked with *Coccidioides* would recommend the mouse for diagnostic work Second intranasal challenge of the mouse provides a more rigorous test in experimental chemotherapy and experimental immunization Furthermore, examination of susceptibility of animal hosts to inoculation by different routes may be of importance in the development of immunization with viable organisms, as has been attained by subcutaneous inoculation[25] Perhaps the innocuousness of *C immitis* by the gastrointestinal route could lead to examination of this as well as parenteral routes in experimental immunization

Acknowledgments

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MECHANISMS OF ACTION OF ANTIBIOTICS*

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The discovery and development of antibiotics have brought about a new era in medical and biological sciences Tuberculosis which was responsible for 45 2 deaths/100 000 citizens of the United States in 1939 caused only 5 deaths/100 000 in 1957 Mortality resulting from acute rheumatic fever appendicitis and syphilis has also decreased sharply during this period (3 5 to 0 5 9 9 to 1 2 and 11 2 to 2 2 per 100 000 citizens of the United states respectively)[1] The widespread use of penicillin streptomycin the tetracyclines and other antibacterial antibiotics has been responsible to a great extent for these dramatic reductions Effective antifungal antibiotics have been developed only recently partially because severe mycotic infections are less frequent and less contagious than grave bacterial infections Moreover the prevalence of fungus diseases has been underestimated, it is seldom realized that North American blastomycosis, candidiasis, cryptococcosis and aspergillosis cause as many deaths as tetanus salmonellosis chicken pox and bacillary dysentery In 1955 aspergillosis blastomycosis bacillary dysentery salmonellosis and tetanus each was responsible for the death of 2 Minnesotans candidiasis was responsible for 3 deaths In 1956 cryptococcosis and chicken pox each was the cause of 2 deaths in Minnesota[2] In addition the incidence of candidiasis has been increasing especially following prolonged treatment with broad spectrum antibacterial antibiotics[3] Cancer patients, particularly those receiving X ray therapy, and diabetics seem to be more susceptible to mycoses than the population as a whole[4, 5]

Nystatin the first clinically accepted antifungal antibiotic, alters favorably the course of candidiasis[6] but not of dermatophytoses or deep seated mycoses[7] Clinical results reported in this publication indicate that griseofulvin and amphotericin B are becoming firmly established as effective drugs of choice for dermatophytoses and deep mycoses respectively (J H Seabury H F Dascomb and F J Roth) Unfortunately our current understanding of the mode of action of these antibiotics is limited This discussion therefore reviews briefly known and postulated mechanisms of action and outlines our approach to this problem using *Candida stellatoidea* and nystatin as the test system In order to elucidate the mode of action of an antibiotic the cellular function(s) affected by the drug must be determined Possible physiological loci include (1) protein synthesis (2) nucleic acid synthesis (3) synthesis or activity of coenzymes (4) energy generation or transfer (5) permeation (6) cellular and subcellular organization and (7) the metabolic self regulating feedback system The forenamed categories can not always be delineated sharply for example anabolism and catabolism are dependent on protein (enzyme) synthesis A similar series of interactions involves active transport of precursor fabrication of complex molecules and integration of these into cellular structure and function all of these steps require abundant energy

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Nature of the Antifungal Activity of Nystatin

R. Brown and E. Hazen (elsewhere in this monograph)[8] have noted that 1.5 to 13 μg nystatin/ml prevented growth of a wide variety of yeasts and molds but that higher levels were fungicidal. Similarly we have found that as little as 1 μg/ml inhibited growth of *C. stellatoidea* completely and that 0.5 μg/ml decreased the final crop of cells (TABLE 1). As the nystatin in the growth medium lost potency the yeasts exposed to an initial concentration of 1 μg/ml began to grow. Viable cell counts revealed that even after 48 hours exposure to the drug at an initial concentration of 2 μg/ml a significant number of fully sensitive persistors could be recovered. When 4 μg nystatin/ml of nutrient medium was added to a population of *C. stellatoidea* in the exponential phase of growth there was an immediate increase in turbidity followed by cessation of growth (FIGURE 1). If the *p*H of the milieu

TABLE 1

EFFECT OF NYSTATIN ON GROWTH AND SURVIVAL OF *CANDIDA STELLATOIDEA*

Nystatin concentration μg/ml	Viable count yeasts/ml		
	0 time	24 hours	48 hours
0	6.9 × 10^4	1.5 × 10^8	3 × 10^8
0.5	6.9 × 10^4	5 × 10^7	3 × 10^8
1	6.9 × 10^4	5.4 × 10^6	1.1 × 10^8
2	6.9 × 10^4	2.5 × 10^6	2.6 × 10^4
3	6.9 × 10^4	6 × 10^3	4 × 10^3
4	6.9 × 10^4	<10^2	<10^2

Cultures were incubated at 30°C on a reciprocal shaker

was maintained above 4.5 the effect of nystatin was fungistatic; conversely if the *p*H of the medium was permitted to fall below 4.5 the antibiotic was fungicidal. The primary action of this drug therefore seemed to be suppressive rather than lethal. The latitude between inhibitory and destructive concentrations was very narrow however and dependent on the chemical and physical environment. While studying the mode of action of nystatin it is well to keep in mind that fungicidal and fungistatic manifestations of the interactions among cell environment and drug may proceed simultaneously. The physiological locus (loci) for the two effects may be identical sequential or diverse.

Tests for Indirect Action

A drug need not interact directly with the susceptible cell in order to exert its deleterious effect. An antibiotic can conceivably react with essential nutrients in the medium thereby rendering them unavailable to the cell. A chelating agent for example may deplete the milieu of needed calcium ions. Inasmuch as nystatin exerted progressively greater antifungal activity at increasing insoluble levels its primary effect may have involved extracellular chelation. Because nystatin is only slowly dialyzable[9] this hypothesis was tested directly. To double strength growth medium[10] 8 μg nystatin/ml was

added After 2 hours' incubation at 30° C, 10 ml of this medium was transferred to a dialyzing tube, the sterile bag was placed aseptically into 10 ml deionized water After 2 more hours the dialyzing chambers were removed and the dialysate was inoculated with *C stellatoidea* Growth in dialyzed

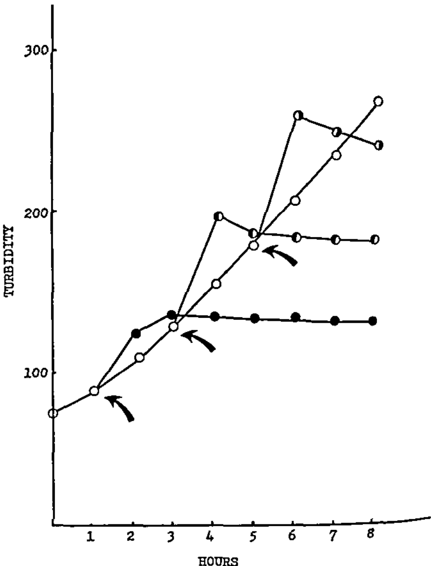


FIGURE 1 Effect of nystatin on growth of *Candida stellatoidea* To cultures in the exponential phase of growth (○) 4 μg nystatin/ml was added (as indicated by arrows) at hour 1 (●) hour 3 (◐) or hour 5 (◑) Growth was measured with a Klett Summerson photoelectric colorimeter (filter 42)

nystatin treated medium was identical to growth obtained in dialyzed but untreated medium The primary effect of the drug, therefore, was not on the composition of the nutrient substrate This experiment did not rule out the possibility that nystatin leached an essential component from the cells To test this hypothesis, 10 ml of growth medium containing 40 to 400 µg antibiotic was dispensed into dialyzing bags The bags were transferred to flasks that held 20 ml growth medium The drug free milieu was inoculated with *Candida*, growth was equivalent in control cultures and the experimental series Nystatin, therefore, did not leach the yeasts of dialyzable, essential nutrients

TABLE 2
INFLUENCE OF HYDROGEN ION CONCENTRATION ON FUNGICIDAL ACTIVITY OF NYSTATIN

*p*H	Survivors/ml		
	0 nystatin	4 µg nystatin/ml	40 µg nystatin/ml
1	5×10^5	3×10^3	10^2
2	5×10^5	5×10^2	$<10^2$
3	5×10^5	4×10^3	10^2
4	5×10^5	1×10^5	10^2
5	5×10^5	2×10^5	5×10^4
6	5×10^5	2×10^5	$1\ 5 \times 10^4$
7	5×10^5	3×10^5	2×10^4
8	5×10^5	3×10^5	2×10^4
9	5×10^5	3×10^5	3×10^4
10	5×10^5	3×10^5	2×10^4
11	5×10^5	2×10^5	4×10^3

Yeasts were treated in growth medium for 2 hours at 30°C

Binding

In a series of enlightening studies, Lampen *et al* [9, 11] have shown that nystatin is absorbed (or adsorbed) by yeasts "Absorption" of antibiotic was found to be dependent on hydrogen ion concentration, temperature, and availability of energy Their findings and ours, using viability as the index of drug activity, are parallel *C stellatoidea* survived exposure to 4 µg nystatin/ml best at neutral or alkaline *p*Hs, maximal killing occurred at *p*H 2 (TABLE 2) Over the range of *p*H 3 to 9, Lampen *et al* found that absorption was maximal at *p*H 3, unfortunately drug uptake at *p*H 1 and 2 was not determined They also demonstrated that baker's yeast bound little nystatin at 4° C, and that cell death was negligible Our findings with *C stellatoidea* confirmed their observations at *p*H 2 5, 4 µg nystatin/ml rendered 90 per cent of the plating units nonviable after 60 min at 30° C, but at 0° C, more than 90 per cent of the treated cells remained viable (TABLE 3) Moreover energy was required for killing as well as for binding Starved *Candida* cells in buffer adjusted to *p*H 2 5 were not harmed by nystatin but when glucose was added survival dropped precipitously (TABLE 4)

Prophylaxis

It has been reported that cysteine,[12] serum,[13] and bile salts[14] reduced substantially the power of nystatin to inhibit growth of *C. albicans*. Additionally Lampen *et al.*[11] demonstrated that zymosan and serum prevented binding and Bradley[15] found that inorganic phosphate protected glycolysis from inhibition by nystatin, but did not affect fungicidal activity of the drug. We have screened several substances for capacity to protect *Candida* from nystatin

TABLE 3
EFFECT OF TEMPERATURE ON FUNGICIDAL ACTIVITY OF NYSTATIN

Temperature (°C)	Survivors in 4 μg nystatin/ml.			
	0 time	30 min	60 min	120 min.
0	5×10^4	5×10^4	4.5×10^4	4.5×10^4
10	5×10^4	2.5×10^4	2×10^4	5×10^3
20	5×10^4	7.5×10^3	5×10^3	2.5×10^2
25	5×10^4	6.5×10^3	3.6×10^2	2.1×10^2
30	5×10^4	6.1×10^3	4.1×10^3	1.7×10^2
37	5×10^4	6.3×10^3	4.8×10^3	2.3×10^2

Yeasts were treated in growth medium adjusted to *p*H 2.5

TABLE 4
EFFECT OF NYSTATIN ON ENERGY DEPRIVED CELLS

Treatment of cells	Suspending milieu	Atmosphere	Survivors
Washed 3 times	Buffer	Air	5×10^6
Washed 3 times	Buffer	N_2	5×10^6
Washed 3 times	Buffer + nystatin	Air	4×10^4
Washed 3 times	Buffer + nystatin	N_2	4.8×10^5
Washed & starved	Buffer	Air	5×10^5
Washed & starved	Buffer + nystatin	Air	5×10^6
Washed & starved	Buffer + glucose	Air	5.6×10^6
Washed & starved	Buffer + glucose + nystatin	Air	3×10^4

Candida stellatoidea was starved by shaking washed cells in *M*/15 phosphate buffer *p*H 4.5, for 4 hours at 30° C. Milieu was adjusted to *p*H 2.5 and maintained at 30° C. Yeasts were exposed to 4 μg nystatin/ml. and to 4 mg glucose/ml., when indicated, for a period of 2 hours.

toxicity. Only serum, oxgall, nucleinic acid, vitamin E, ascorbic acid, cysteine sodium thioglycolate, and glutathione were able to protect at the concentrations tested (TABLES 5 and 6). These substances were required in amounts far in excess of growth-factor levels. It should be noted that most of these substances lower the oxidation reduction potential (Eh) of the milieu, the facts that an increase in *p*H usually results in a lowering of Eh,[16] and that fungicidal potency becomes more marked as *p*H decreases are consistent with the hypothesis that Eh is inversely correlated with killing power.

Permeability

One of the cell's most vulnerable sites is the plasma membrane. Failure of selective permeability leads to loss to the environment of vital components of

the cell and allows qualitatively and quantitatively deleterious materials to enter the protoplasm We have attempted to determine whether nystatin acts by disrupting cell permeability by correlating dye uptake and viability

TABLE 5
ADDITIVES UNABLE TO PROTECT *CANDIDA STELLATOIDEA* AGAINST NYSTATIN TOXICITY

Alkali inactivated nystatin‡	Margaric acid
Ascorbic acid	Methanol
Brucine alkaloid	Mercaptobenzthiazole
5 bromo-2 4 dihydroxypyrimidine	Nickelous sulfate
n Caprylic acid*	Nystatin sensitive methanol killed *Candida stellatoidea*†
n Caproic acid	Nystatin resistant methanol killed *Candida stellatoidea*†
Cadmium chloride	Oxgall
Calcium chloride	Phenylhydrazine HCl
Citric acid	Sitosterol
L cysteine HCl	Sodium arsenate
Dibutyl phthalate	Sodium cyanide
Disodium ethylenediaminetetraacetic acid	Sodium fluoride
2 ethoxy 6 9 diaminoacridineazosulfanilamide	Sodium hydrosulfite
Ferrous sulfate	Sodium thiocyanate
Formamide	Stannic chloride
Ghatti gum	Thallium chloride
4-hydroxy 2 thiopyrimidine	Thioflavin TG*
Indole-3 acetic acid	Tween 80
Keratin	Uracil
Lithium chloride	2% vitamin E
Magnesium sulfate	Zinc oxide
Manganous sulfate	Zymosan

Nutrient substrate contained 4 µg nystatin/ml and 5 or 50 µg additive/ml
* Fifty micrograms additive/ml was toxic to *Candida stellatoidea* in the absence of nystatin
† 10^8 and 10^7 dead cells/ml
‡ Heated at 60° C for 10 min at *p*H 9

TABLE 6
ADDITIVES EFFECTIVE IN PROTECTING *CANDIDA STELLATOIDEA* FROM NYSTATIN TOXICITY

Additive	Minimum protective concentration
Sodium thioglycolate	1 mg /ml
(Vitamin E) tocopherol	200 µg /ml
Oxgall	1 mg /ml
Cysteine	100 µg /ml
Ascorbic acid	200 µg /ml
Nucleinic acid	200 µg /ml
Glutathione	5 mg /ml
Human serum	5 mg /ml

Nutrient substrate contained 4 µg nystatin/ml

If this antibiotic destroys selective permeability, stain should enter the cell concurrently with or prior to cell death as measured by viable counts Using 6 different dyes we found that viability in 4 µg nystatin/ml at *p*H 2 5 dropped concurrently with or before dye penetration was detected (TABLE 7) The discrepancy between dye uptake and loss of viability was even more striking

with 4 μg drug/ml at pH 3 5, where killing proceeded more slowly Methylene blue and crystal violet, for example, each stained only 5 per cent of the cells after 30 min exposure to the antibiotic, but less than one half of the population was viable after 1 hour, 12 per cent of the population was viable but 90 per cent of the yeasts were unstained, after 2 hours, only 5 per cent of the cell were able to produce colonies, however, 75 per cent remained unstained These data suggest that nystatin does not destroy selective permeability completely but may alter specifically active tran port of a limited class of substances The latter alternative is supported by the observations of Gottlieb *et al* [17] who have studied filipin another polyene antibiotic [18] It seems reasonable to compare mechanisms of action of filipin, nystatin, and amphotericin directly inasmuch as they are similar chemically Moreover mutants of *C stellatoidea* selected

TABLE 7
CORRELATION OF VIABILITY AND DYE UPTAKE

Time in minutes	Per cent stained cells						Viable count	Per cent kill
	Methylene blue	Crystal violet	Janus green B	Safranin O	Neutral red	Trypan blue		
0	1	1	2	3	1	1	5×10^5	0
15	78	78	82	3	1	4	1×10^5	80
30	88	83	82	68	6	7	8×10^4	84
60	92	87	87	72	12	29	5×10^4	90
90	95	95	92	95	25	75	3×10^4	94
120	99	99	99	99	60	99	2×10^4	96

Yeasts were treated with 4 μg nystatin/ml at pH 2 5 incubation temperature was 30 C. Dyes were used at a concentration of 250 μg /ml except for crystal violet that was at 25 μg /ml

for resistance to nystatin manifested increased resistance to amphotericin B and filipin By measuring oxygen consumption manometrically, Gottlieb *et al* found that filipin inhibited oxidative utilizations of glucose and sodium acetate by *Saccharomyces cerevisiae* Surprisingly, they noted that respiratory capability of cell free preparations was not suppressed by this antibiotic Their results can be interpreted as evidence that polyene antibiotics act by preventing permeation of organic carbon compounds rather than by inhibiting the degradative enzymes directly

Energy Generation and Transfer

Nystatin inhibits oxidative and fermentative utilization of glucose and several other energy sources [19, 20] Glycolysis by *C stellatoidea* was suppressed most at pH 2,[21] which correlated precisely with our observation that greatest fungicidal activity was manifested at pH 2 However, energy generation does not seem to be the primary site of nystatin activity because aerobic utilization of sodium glutamate and sodium citrate was unaffected by the drug yet growth would not proceed in medium in which citrate or glutamate replaced glucose as the principal energy source (TABLE 8) Moreover glycolysis by a nystatin resistant variant, which grew in milieu containing 20 μg antibiotic/ml was as susceptible to nystatin as the sensitive parental strain

Protein and Nucleic Acid Synthesis

As our understanding of biosyntheses expands, it becomes increasingly evident that ribonucleic acid plays an important role in the fabrication of proteins. Simply stated, amino acids individually are "activated" by adenosine triphosphate, each amino acid is then transferred to a soluble ribonucleic acid molecule, the amino acid soluble ribonucleic acid complex then attaches specifically to the ribonucleoprotein template of the microsome, there the amino acids join, by peptide bonds, to form proteins, the soluble ribonucleic acid then floats free of the template and is available for another cycle[22]. The specificity of the ribonucleoprotein template is determined by the "genetic material," that is, the desoxyribonucleoprotein. It is difficult, therefore, to differentiate between a primary effect on protein synthesis, ribonucleic acid synthesis, and desoxyribonucleic acid synthesis.

TABLE 8
EFFECT OF NYSTATIN ON GROWTH AND RESPIRATION OF *CANDIDA STELLATOIDEA* IN SODIUM CITRATE AND SODIUM GLUTAMATE

Carbon source	Growth (turbidity)		Respiration (O_2 uptake)	
	Control	1 μg nystatin/ml	Control	1 μg nystatin/ml
Basal medium only	35	0	9	22
Sodium glutamate	110	0	165	145
Sodium citrate	90	0	186	167
Glucose	325	0	259	12

Basal medium was growth medium lacking glucose. 10 mg./ml. citrate or glutamate was added when indicated. Cultures were incubated for 18 hours at 30 C. growth was measured turbidimetrically with a Klett Summerson photoelectric colorimeter (filter 42). Oxygen uptake was measured manometrically at pH 4.5. vessels contained 100 mg. carbon source and 5 mg. dry weight of cells in M/15 phosphate buffer.

Primary suppression of protein fabrication can occur by: (1) interference with amino acids synthesis, (2) prevention of permeation of exogenously required amino acids, (3) inhibition of amino acid activation, (4) interference with the amino acid soluble ribonucleic acid complex, or (5) prevention of attachment of the complex to the template. Chloramphenicol, at bacteriostatic concentrations, does not inhibit oxidation or fermentation of glucose, permeation of amino acids, ribonucleic acid or desoxyribonucleic acid synthesis, amino acid activation, or synthesis of simple peptides, but does inhibit inducible enzyme formation and general protein synthesis[23,24]. Present evidence indicates that chloramphenicol forms an inactive complex with the transfer (soluble) ribonucleic acid. Cycloheximide, like chloramphenicol, inhibits protein synthesis, but does not inhibit respiration, fermentation, or ribonucleic acid synthesis. Cycloheximide, unlike chloramphenicol, does inhibit desoxyribonucleic acid synthesis[25]. Amphotericin likewise fails to suppress oxidative metabolism but it does inhibit protein synthesis and nucleic acid synthesis (E. Drouhet *et al.*, elsewhere in this monograph)[26]. Results with nystatin are not as clear cut, it is obvious that phosphate metabolism is affected by this drug, but the exact physiological locus (or loci) is not known[20,27]. There is

suggestive evidence that suppression of protein synthesis by nystatin is a secondary effect.[20] However, nystatin at concentrations that do not affect growth of *Penicillium chrysogenum* does inhibit production of the enzyme amylase.[25]

Effects on Coenzymes

There are two general classes of coenzymes: the vitamins and the heavy metals. Heavy metals, as cations, are required in trace amounts by all cells. The tetracycline antibiotics are chelating agents whose antibacterial toxicity can be reversed, in large part, by manganic ions. Enzymes isolated from chlortetracycline sensitive and resistant bacteria were found to differ in the firmness with which the metal was bound to the apoenzyme; sensitivity was correlated with ease of dissociation.[29]

Studies on the mode of action of chemotherapeutic agents may contribute substantially to our understanding of basic biology. Perhaps the best example of this is the work with the sulfonamides that led to the discovery of para amino benzoic acid. It is generally accepted today that sulfonamides exert their growth inhibitory action by competing for an enzyme that catalyzes the metabolism of this vitamin. Folic acid, which is the product of this reaction, is the coenzyme involved in the transfer of one carbon residues.[30]

Effects on Structure

The cell wall has proved to be a sensitive target for antibiotic activity because microbes with aberrant outer membranes die quickly in most environments. Moreover, bacterial cell walls contain substances not found in mammals (muramic acid, D-alanine, D-glutamic acid, and diaminopimelic acid);[31] therefore, drugs having detrimental effects on the cell wall will possess minimal toxicity for the host. *Penicillin is the best known agent that interferes with* synthesis of the outer membrane. At low concentrations of antibiotic, sensitive bacteria grow as long filaments, at higher concentrations, the cells become spherical and lyse. In hypertonic media, the cells form osmotically fragile protoplasts that resemble L forms.[32] It is apparent that penicillin interferes in some way with polymerization of cell wall material. Similarly, griseofulvin affects plasticity of the cell wall of fungi. This antibiotic, also produced by members of the genus, *Penicillium*, causes stunting, branching and spiral twisting of the hyphae in fungi having chitinous cell walls, but does not affect molds with cellulosic cell walls.[33] Another fungistatic antibiotic, designated only as the bulging factor, induces hyphal distortion.[34] It is not known whether the action of the bulging factor is on the biosynthesis of cell wall material or upon water balance.

Effects on Metabolic Control

Conceivably, microbes can be killed, not by inhibiting an essential synthesis but by stimulating a synthesis. An artificial system employing a thymineless prolineless mutant of *Escherichia coli* may be used as an example. In medium lacking the two growth factors, Barner and Cohen found that about 50 per cent of the population died within 3 hours; if proline was added, less than 1 per cent

of the population was viable after 3 hours' incubation Even though survival dropped sharply in medium containing proline but deficient in thymine, there was a substantial increase in cellular material, as measured turbidimetrically [85] It may be possible to contrive conditions in which a drug that normally suppresses growth will be germicidal For example, sulfonamide inhibition can be reversed by para amino benzoic acid, or by a mixture of substances whose synthesis is mediated by folic acid (amino acids, panthothenic acid, and thymidine) [80] In the presence of such compounds as sulfonamide, amino acids, vitamins, and purine nucleotides but in the absence of thymidine, cells may be killed more rapidly Alternatively, nonutilizable inducers of adaptive enzymes can conceivably make stringent demands on the supply of activated amino acids, thereby stopping essential syntheses

Effects on Lipids and Sterols

Although few microorganisms are known to have exogenous lipid or sterol requirements, these substances undoubtedly play an important role in cellular metabolism and integration Gottlieb *et al* have suggested that filipin might prevent the synthesis of sterols necessary for growth or competitively replace sterol cofactors in essential metabolic steps [86] The finding that led to this intriguing hypothesis was that cholesterol, ergosterol, sitosterol, and stigmasterol protected *Hansenula subpelliculosa* from the toxic effects of filipin It should be noted, however, that the possibility of an extracellular interaction between the sterols and filipin has not yet been conclusively eliminated Another inhibitor of fungi, hexachlorocyclohexane, seems to be antagonistic to meso inositol,[87] which is a constituent of phospholipids [88] Analogues, either of chemical or biological origin, may prove to be useful chemotherapeutic agents Test systems employing lipoic acid with *Streptococcus faecalis* or divalonic acid with *Lactobacillus acidophilus* are awaiting exploitation

Approaches to the Study of Mechanisms of Action of Antibiotics

It is necessary to determine which, if any, of the major synthetic systems are affected by a drug (protein, ribonucleic acid, or desoxyribonucleic acid synthesis) Effects on the energy generation system and on over-all cellular integrity as determined cytologically should be examined Products accumulated within the cell or milieu should be analyzed qualitatively and quantitatively Based on this information and on data from comparative studies on intact cells and cell free preparations, deductions about permeation can be made Knowledge about the fate of an antibiotic, that is, its binding site, provides much insight into its action Materials that protect against or reverse the toxicity of an antibiotic should be sought Comparative studies using the sensitive parental strain and resistant variants help locate specifically the site of action Moreover, cross resistance patterns, with respect to other drugs and metabolic poisons, may point out similarities with better-known models Interactions between drug substrate, and isolated enzyme systems may reveal definitively the physiological locus affected by a drug Methodology for the recognization of primary effects on cellular integration and metabolic control is still relatively crude To date the mode of action of few

antibacterial antibiotics and no antifungal antibiotic is well understood We are confident that better understanding of drug action will lead to improved therapeutic procedures and that new, superior chemotherapeutic agents may be developed as a result of such work

Summary

The success of antibiotics that kill or suppress pathogenic microbes should not cause us to ignore other avenues leading to the control of infectious diseases Drugs that prevent synthesis of toxins or inhibit production of enzymes needed for invasion may be equally effective therapeutic agents Manipulation of the genetic make up of the parasite, by transduction, transformation or other genetic processes, may enable us to convert highly virulent germs into harmless saprophytes Drugs aimed at the host rather than the intruding microbe are in order The defense mechanisms of man frequently wall-off the pathogen, protecting it from effective antibiotics Reducing the host's response by adrenocorticotrophic hormone (ACTH), for example, has proved to be a useful adjunct in the treatment of tuberculosis Moreover, the reaction between the antibody forming system and an antigen may be too efficient leading to hypersensitivity or to immunological paralysis of the host, that is, inability to produce antibodies against the antigen If immunological paralysis can be prevented protective antibody may provide adequate resistance to disease Drugs stimulating production of phagocytes and antibody synthesizing cells may be practicable Nevertheless, the search for new antibiotics, particularly against fungi, viruses, and cancer, and the modification of existing ones must continue

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Part III. Immunology of Mycoses

SOME ASPECTS OF THE MODE OF ACTION OF POLYENE ANTIFUNGAL ANTIBIOTICS

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Introduction

Since the discovery of nystatin in 1951 by Hazen and Brown,[7] a new class of antifungal agents has been described in recent years, most of these are products of various *Streptomyces* fermentations These agents, characterized by a chromophore group formed by conjugating double bonds, are grouped under the name of polyene antibiotics;[39] they exhibit characteristic ultraviolet absorption spectra and are of low solubility in water, but dissolve more readily in aqueous solutions of the lower alcohols or pyridine They inhibit the growth of a wide range of fungi including yeasts and filamentous fungi, pathogenic or non pathogenic, but are ineffective against bacteria, actinomycetes, and viruses or animal cells, they are not toxic when given by mouth, but show some toxic side effects, such as hyperthermia, when injected into animals and humans [13,14] The important success of the polyene antibiotics, such as nystatin[7] and amphotericin B[23,54] in the treatment of mycotic infections, focused the attention of some workers on the mode of action of these antibiotics and the factors contributing to their activity [3-4,14,20,24,28,33,34,40,52]

The key to successful chemotherapy is contained in the selective toxicity, the antibiotic is more toxic to the pathogen than to the host [1,19] If a chemotherapeutic agent acts by inhibiting an enzymatic system of fundamental *importance to the pathogen, then this system must be absent from the* host or if present, less susceptible to the drug [53] The discovery of the site of primary injury of antibiotics is not easy, as Hotchkiss pointed out ten years ago [28] He compared the investigator to an expert "trying to examine a crashed airplane for evidences of the initial mechanical failure which led to the accident, since the mechanical system is now grossly disrupted and he does not understand it very well anyway, he naturally places greater weight upon any testimony obtainable as to events before the crash, the earlier the better"

The first study on the effect of polyenes on the fungal metabolism was the work of Lampen *et al*,[32,33] who established the fact that nystatin inhibits the endogenous respiration and the aerobic and anaerobic utilization of glucose by yeasts and other fungi, however, these authors observed that with certain levels of nystatin there is an initial stimulation of oxygen consumption, then metabolism ceases abruptly Bradley[2,4] showed that nystatin as well as arsenate inhibited the glucose fermentation of *Candida stellatoidea* However, several different workers, including Harman and Masterson[24] with nystatin, and Fujii[19] with trichomycin in their experiments attained the stimulation of the respiration of *Candida albicans* and *Trichophyton*

Our preliminary work[14] on the action of amphotericin B on the respiratory metabolism of *C albicans*, supplemented with further investigations on other polyene antibiotics, emphasized the fact that one of the primary effects of the

polyenes is the stimulation of O_2 uptake the inhibition of respiration being the final result of the death of the cells. The analogy of the action with dinitrophenol, an agent that stimulates the cellular oxidations and controls the respiratory rate by processes related to oxidative phosphorylation, led us to extend our work to the effect of amphotericin B on the phosphorus metabolism and on the nucleic acids and protein synthesis during the growth of *C albicans*.[25,28] All these aspects will be developed in this paper.

TABLE 1
DATA ON THE ANTIBIOTICS STUDIED

Antibiotic* producing organism	Type of polyene $(CH{=}CH)_n$	Action on *C albicans* (st AB)			
		Fungistatic effect MIC/ml/24 hours†	Fungicidal effect 4×10^7 yeasts/ml = 1 mg dry weight % lethality		
Amphotericin B *Streptomyces* sp	Heptaene	0.2 µg	50 µg 1.5 µg	pH 7.1 15 20 6 hours pH 4.1 6 hours	 30% 50% 75% 90%
Ascosin *Streptomyces canescus*	Heptaene	0.1 µg	3.12 µg 6.25 µg.	pH 7.1 6 hours 6 hours	 47% 90%
Amphotericin A *Streptomyces* sp	Tetraene	3.12 µg			
Nystatin *Streptomyces noursei*	Diene-tetraene	3.12 µg			

* Potency of the antibiotics used amphotericin B (lot DIAM 8 24CR) 850 U/mg amphotericin A (lot HV 733) 995 U/mg nystatin (commercial sterile powder) 2500 U/mg ascosin (lot R16 and R17). The first 3 antibiotics furnished by the Squibb Institute for Medical Research New York N Y ascosin by Commercial Solvents Corporation New York, N Y. Five to 10 mg of each antibiotic dissolved in 0.2 ml of dimethylacetamide followed by appropriate dilution in water and buffer solution.

† Minimal inhibitory concentration/ml of the following synthetic medium glucose 2 gm $SO_4(NH_4)_2$ 1 gm $PO_4HNa_2 + 12H_2O$ 3.34 gm PO_4H_2K 0.54 gm $SO_4Mg + 7H_2O$ 0.25 gm biotin 10^{-8} trace elements distilled water 1000 ml. Continuous agitation 34° C 3 × 10⁶ yeasts/ml initial concentration.

Action of Polyene Antibiotics on the Respiratory Metabolism

Some representative data on the antifungal antibiotics studied are given in the TABLE 1. The fungicidal effect on *C albicans* is measured under the same conditions as those used in Otto H Warburg's techniques on 1 ml of a suspension of yeasts representing 4×10^7 cells (equivalent to 1 mg dry weight). Fifty µg of amphotericin B the usual concentration utilized in the respiratory studies at *p*H 7 kills 30 per cent of the cells after 15 min 50 per cent after 20 min and at the end of the experiments using Warburg flasks after 6 hours this concentration kills 75 per cent of the cells. At an acid *p*H only 1.5 µg is required to kill 90 per cent of the cells after 6 hours. This is in accordance with the results of Lampen *et al*[14] on the influence of *p*H on the fungicidal effect

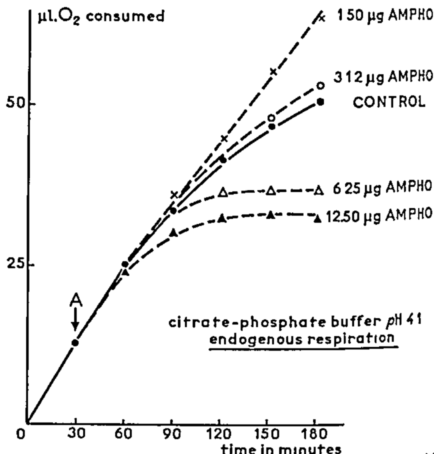


FIGURE 3 Effect of amphotericin B on the endogenous respiration of yeasts suspended in an acid buffer solution (McIlvaine citric acid phosphate buffer *p*H 4 1) Growing yeasts after 16 hours incubation, in the same concentration as in FIGURE 1 Amphotericin B (final concentrations noted on the curves) added where shown by the arrow

TABLE 3

ACTION OF AMPHOTERICIN B ON THE RESPIRATION OF *C ALBICANS* AT *p*H 4 1

Time (min)	Control			Amphotericin B 1 5 µg /ml.			
	O_2 (µl)	CO_2 (µl)	RQ	O_2 (µL)	%	CO_2 (µl)	RQ
	Endogenous respiration						
30	13 13	14 29	1 08	Antibiotic at 30 min			
60	21 90	22 87	1 04	20 08	−8	19 09	0 95
120	34 13	35 57	1 04	35 22	+3	32 70	0 92
180	43 56	44 32	1 01	52 83	+21	49 19	0 93
	Exogenous respiration—1% glucose						
30	21 87	32 99	1 50	Antibiotic at 30 min			
60	41 61	49 31	1 18	48 36	+16	62 30	1 28
120	93 09	123 87	1 33	101 59	+9	127 18	1 25
180	132 22	171 86	1 29	131 90	−0 2	164 19	1 18

Citrate phosphate buffer *p*H 4 1, 1 ml of growing cells (1 mg dry weight) per flask Citric acid—Na_2 phosphate Key +, per cent stimulation, −, per cent inhibition

μg amphotericin B as shown in FIGURE 3 and in TABLE 3 These results are explained by the fungicidal effect, which is greater at an acid *p*H

The O_2 uptake of the washed yeasts after contact with amphotericin B for various lengths of time is not stimulated as are normal yeasts in the presence

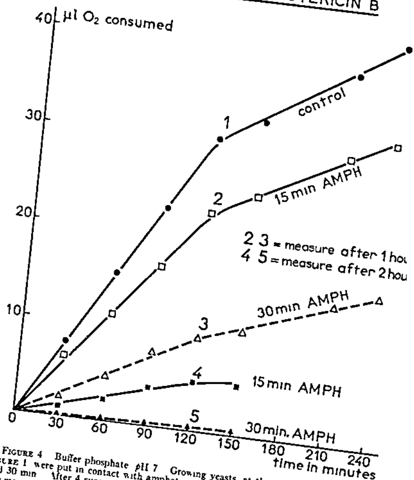


FIGURE 4 Buffer phosphate *p*H 7 Growing yeasts at the same concentrations as in FIGURE 1 were put in contact with amphotericin B (50 μg/ml final concentration) for 15 and 30 min After 4 successive washings with distilled water the O_2 uptake of the yeast was measured under the same conditions as in the experiments made in the presence of amphotericin B The same final concentration was attained in both experiments Curves 2 3 measurement of respiration 1 hour after washing 4 5 measurement after 2 hours

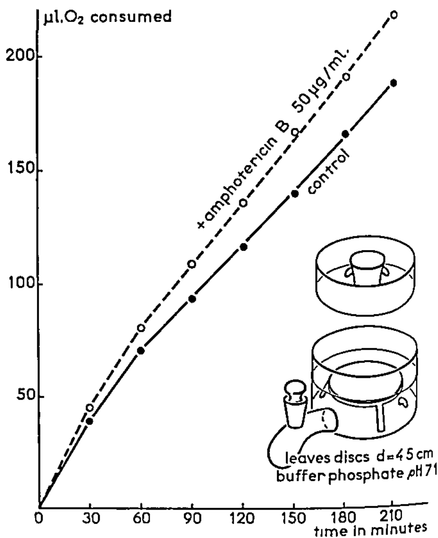


FIGURE 5 Discs of tobacco leaves 4.5 cm diameter immersed in amphotericin B (100 µg/ml of buffer phosphate *p*H 7.1) or in buffer phosphate (control) for 4 hours before respiration, O_2 uptake studied in special flask of 30 ml Endogenous respiration

of the same concentrations, it is strongly inhibited, and the inhibition is a function of the duration of the contact The data from one of these experiments on the respiration of washed yeasts after contact with amphotericin B at *p*H 7 are summarized in FIGURE 4.

The experiments prove that the amphotericin B penetrates rapidly into the cells even after 6 successive washings the antibiotic is still found in the cells It is possible that the washings eliminate the products given off by the yeasts that were submitted to the action of the antibiotic These products may be

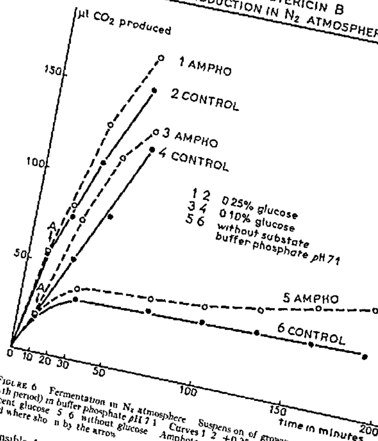


FIGURE 6 Fermentation in N_2 atmosphere Suspension of growing yeasts (16 hours growth period) in buffer phosphate *pH* 7 1 Curves 1 2 +0 25 per cent glucose, 3 4 +0 10 per cent glucose 5 6 without glucose Amphotericin B (50 µg /ml final concentration) added where shown by the arrow

responsible for stimulation of the O_2 consumption A loss of essential metabolites and phosphorylated compounds diffusing out of the cells after contact with nystatin was observed independently by Osteux and his co workers[40] and by Scholz *et al*[46] These authors showed that the disorder in the permeability of the cell membrane takes place within a few minutes after the addition of the antibiotic Some disorders in the membrane permeability accompanied by a

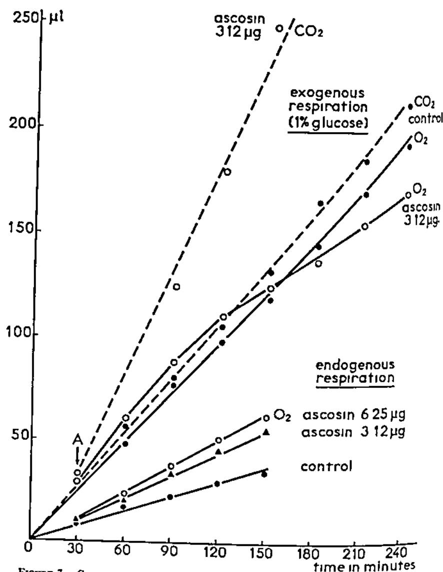


FIGURE 7 Growing yeasts in 0 01 *M* phosphate buffer *p*H 7 same conditions as in FIGURES 1 and 2 Ascosin at various concentrations added where shown by the arrow

release of soluble cell constituents have also been observed on fungi submitted to different antifungal agents [8, 10, 21, 60, 61]

Amphotericin B stimulates the respiration of both fungi and plant tissues The O_2 consumption of discs of tobacco leaves immersed in amphotericin B for

4 hours is stimulated to the order of 16 to 20 per cent at *p*H 7, as is shown in FIGURE 5

In an atmosphere of nitrogen, in the presence or the absence of glucose at *p*H 7, CO_2 production is also stimulated In the absence of carbon substrate this stimulation may rise to 66 per cent and, in the presence of glucose, to 20 o 40 per cent (FIGURE 6)

The stimulation of oxidations is also observed with the other polyenes studied 'IGURE 7 illustrates the results obtained with ascosin, a heptaene antibiotic omewhat more toxic than amphotericin B The stimulation of O_2 uptake at H 7 is obtained with a small concentration such as 3 12 µg With this con- ntration, after an initial stimulation the O_2 consumption is diminished, but e CO_2 production is particularly high, and the R Q rises from 1 30 to 3 16 ABLE 4)

TABLE 4

ACTION OF ASCOSIN ON THE RESPIRATION OF *CANDIDA ALBICANS* AT *p*H 7 1 IN PRESENCE OF 1 PER CENT GLUCOSE

(min.)	Control			Ascosin							
				3 12 µg				6 25 µg			
	O_2*	CO_2*	R Q	O_2	%	CO_2	R Q	O_2	%	CO_2	R Q
30	29 37	38 49	1 31	Addition of the antibiotic at 30 min							
60	48 14	56 53	1 17	55 80	+15	59 51	1 06	60 99	+26	78 37	1 28
90	77 02	80 39	1 04	87 35	+13	124 91	1 42	67 41	−12	156 55	2 32
120	98 21	105 07	1 06	108 93	+10	180 10	1 65	78 96	−19	215 73	2 73
150	119 36	131 14	1 09	122 91	+2	249 24	2 02	91 81	−23	290 61	3 16
180	144 42	178 10	1 23	136 24	−5	331 67	2 43	109 14	−35	367 02	3 36

Phosphate buffer *p*H 7 1, 1 ml of growing cells (1 mg dry weight) per flask.
* Microliter of gas.

A similar stimulation of endogenous and exogenous O_2 uptake of *C albicans* is obtained with tetraene polyenes such as amphotericin A and nystatin (TABLES 5 and 6), the greatest effect is observed on endogenous respiration

In our opinion, the stimulation of O_2 consumption and the rapid oxidation of carbohydrate reserves are the earliest effects of the polyenes on the respiratory metabolism, the inhibition of respiration seems to be a later effect, resulting from the death of the yeasts

The contradiction between the results showing the inhibition of respiration and fermentation[2-4,33,34] and the results showing the burst of metabolic activity[14,20,24] can be explained by the wide range of *p*H (from *p*H 2 0 to *p*H 7 5) employed by these workers in their studies In anaerobiosis, at *p*H 4 5 Lampen *et al*[33] found that yeast glycolysis and oxidation are stimulated by 0 4 µg of nystatin/ml , but strongly inhibited by 3 3 µg /ml Harman and Masterson[24] observed at *p*H 7 a 300 per cent stimulation of O_2 uptake of *C albicans* submitted to the action of nystatin On the other hand, Bradley[4] noted a rapid inhibition of glucose fermentation in anaerobiosis at *p*H 2, but at *p*H 5 6 the fermentation is not inactivated Ribereau Gayon *et al*[43] found that 5 µg

nystatin/ml inhibits strongly the fermentation of glucose by *Saccharomyces ellipsoideus* at *p*H 3.8 to 4.8, but produces an initial stimulation of respiration and fermentation at *p*H 7.

Actidione (cycloheximide), another antifungal antibiotic different from the polyenes, inhibits the endogenous and exogenous oxidations of the sensitive yeasts without any initial stimulating effect, our results on *C. tropicalis* show

TABLE 5

EFFECT OF POLYENES, ANTIBIOTICS, AND DINITROPHENOL ON O_2 UPTAKE OF *CANDIDA ALBICANS*

Time (min.)	Polyenes antibiotics						Dinitrophenol	
	Ascosin		Amphotericin B		Nystatin			
	3.12 μg (%)	6.25 μg (%)	50 μg (%)	100 μg (%)	50 μg (%)	100 μg (%)	50 μg (%)	100 μg (%)
30	+15	+26	+20	+14	+21	+48	+4	+10
60	+13	−12	+18	+10	+30	+37	+5	+12
90	+10	−19	+16	+5	+20	+23	+10	+16
120	+3	−23	+15	0	+16	+8	+10	+15
150	−5	−24	+14	−5	+12	−1	+10	+14

Conditions: buffer phosphate *p*H 7.1, glucose 1 per cent, growing cells of *C. albicans* (1 mg of dry weight) per flask. Key: +, stimulation, −, inhibition of O_2 uptake.

TABLE 6

EFFECT OF POLYENES ANTIBIOTICS AND DINITROPHENOL ON THE ENDOGENOUS RESPIRATION OF *CANDIDA ALBICANS*

Time (min.)	Ascosin		Amphotericin B	Amphotericin A	Dinitrophenol
	3.12 μg (%)	6.25 μg (%)	50 μg (%)	50 μg (%)	50 μg (%)
30	+24	+45	+42	+10	+259
60	+52	+71	+63	+30	+253
90	+62	+85	+73	+36	+280
120	+64	+79	+56	+40	

Conditions: buffer phosphate *p*H 7.1 without substrate, 1 ml. growing cells of *C. albicans* (1 mg. dry weight) per flask.

that actidione, at concentrations varying between 16.2 and 50 μg., has only an inhibitor effect on respiration. The results of Walker and Smith[65] on *Myrothecium verrucaria* and of Kerridge[31] on *Saccharomyces carlsbergensis* are similar. Other antifungal agents affect slightly the respiration of sensitive fungi.[21, 65]

Some stimulatory effects on respiration and on fermentation were observed in the past with low concentrations of various mineral or organic antifungal agents[5, 44, 47] and more recently, with fatty acids on the respiration of dermatophytes.[10] The stimulation of O_2 consumption by antiseptics such as phenol and 2-phenoxyethanol is explained by Hugo[29] by an increase in the permeability

of the microorganisms, thereby facilitating substrate oxidation. An apparent stimulation of oxygen uptake by cyanide is observed in *C. albicans* by Ward and Nickerson [37] and is explained on the basis of its inhibitory effect on catalase activity. The action of most of these agents seems to be different from the effect of the polyenes.

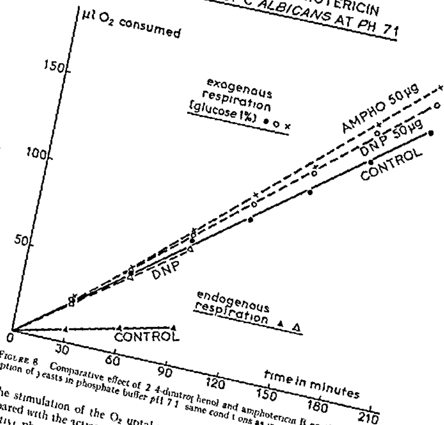


FIGURE 8. Comparative effect of 2,4-dinitrophenol and amphotericin B on the O_2 consumption of yeasts in phosphate buffer pH 7.1; same conditions as in FIGURE 1.

The stimulation of the O_2 uptake of yeasts by polyene antibiotics can be compared with the action of dinitrophenol (DNP), an agent that uncouples the oxidative phosphorylation from the respiration [38,45]. At fungistatic concentration of DNP we obtain the stimulation of the respiration of *C. albicans* (FIGURE 8, TABLES 5 and 6) similar to the stimulation obtained on yeasts,[17,18,41,42,48] molds,[4] bacteria,[41] and mitochondrial preparations of animal cells[38,49] by various authors.

The first observation on the increase of metabolism in animals after the

administration of dinitrophenol or related compounds was reported in 188 by Cazeneuve and Lépine[9] Magne *et al*[37] observed during World War I fata hyperthermia in persons working with DNP and proved its stimulating effec on cellular oxidations in various warm- and cold blooded animals From th point of view the analogy with the hyperthermia produced by the polyene anti biotics must be emphasized The intravenous infusion of therapeutic doses o nystatin (200,000 U/day) or amphotericin B (1 mg/kg/day) may produc a transitory rise of body temperature from 37° C to 40° C

The stimulating effect of DNP on cellular oxidations was extended to plan tissues, yeasts, and molds by Plantefol for the first time in 1931;[41, 42] since tha time various authors have confirmed this work[17, 18, 45] However, some authors

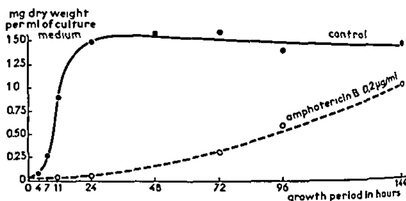
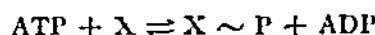


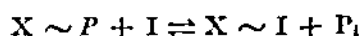
FIGURE 9 Curve of growth of *C albicans* ●——● normal cells O– – –O cells cu tured in presence of 0 2 µg amphotericin B/ml Culture medium as TABLE 1 but with 1 glucose Cultures shaken at 34° C in Fernbach flasks of 400 ml Number of yeasts per m at 0 hour = 3 × 10[6] At the stationary growth period about 8 × 10[7] yeast/ml

observed an inhibitory effect of DNP on respiration, but this effect is explaine by the acid *p*H utilized in their experiments, as for the polyenes, the stimulatin effect of DNP is a function of *p*H and is related to the dissociation of the activ form

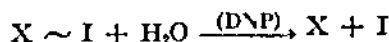
It has been known for more than twenty years that respiration is linked wit the esterification of inorganic phosphate, but it was only in 1948, in the worl of Loomis and Lipmann,[36] that DNP was shown to uncouple phosphorylatio from respiration and to control the respiratory rate DNP seems to stimulat the adenosine triphosphatase (ATPase) activity The DNP stimulated ATPase reactions of liver mitochondria, characterized by *p*H optima at 6 3 7 4 and 8 5, these are explained by the following reactions as proposed by Slater and Hulsmann[49]



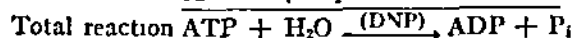
$$ATP + X \rightleftharpoons X \sim P + ADP$$



$$X \sim P + I \rightleftharpoons X \sim I + P_i$$



$$X \sim I + H_2O \xrightarrow{(DNP)} X + I$$



$$\text{Total reaction } ATP + H_2O \xrightarrow{(DNP)} ADP + P_i$$

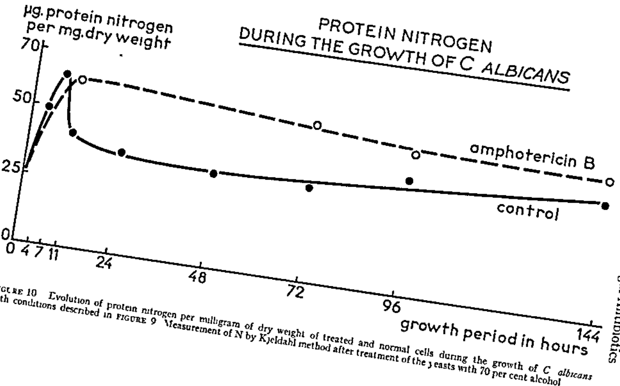


FIGURE 10 Evolution of protein nitrogen per milligram of dry weight of treated and normal cells during the growth of *C albicans*. Growth conditions described in FIGURE 9. Measurement of N by Kjeldahl method after treatment of the yeasts with 70 per cent alcohol.

In this reaction P_i is inorganic phosphate and $X \sim P$ and $X \sim I$ are energy rich intermediates

The adenosine diphosphate (ADP) formed by the action of DNP produces a

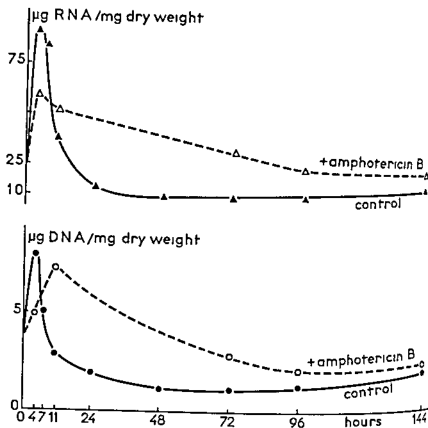


FIGURE 11 Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) measured by spectrophotometry after successive extractions with perchloric acid using the technique of Ogur and Rosen[38]

short increase in the rate of O_2 uptake and finally, the adenosine triphosphate (ATP) regeneration is blocked and vital synthesis compromised

For this reason in the following experiments we tried to relate the effect of amphotericin B on the respiratory metabolism to the effect on other cellular metabolisms such as phosphorus protein, and nucleic acids during the growth of *C albicans* (FIGURE 9)

Action of Amphotericin B on Phosphorus, Protein, and Nucleic Acid Metabolism of C. albicans

FIGURE 10 shows that the treated yeasts are richer in protein nitrogen than the normal cells, but observation of the amounts of protein nitrogen after 6 days when the treated cells are in the exponential period, shows that the synthe-

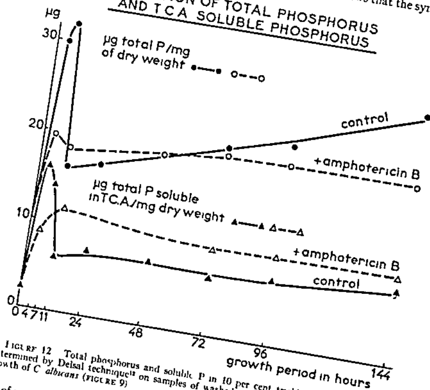


FIGURE 12. Total phosphorus and soluble P in 10 per cent trichloracetic acid (TCA) determined by Delsal technique[18] on samples of washed cells at different times during the growth of *C. albicans* (FIGURE 9).

sis of proteins has been slightly inhibited. Synthesis of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) by the treated cells is slightly inhibited, particularly of RNA, as shown in FIGURE 11. From this point of view, amphotericin B differs from other antifungal antibiotics such as actidione, frequentin, viridin gliotoxin: all antibiotics having little effect on respiration, but a marked inhibitor effect on protein and DNA synthesis and less on RNA synthesis.[6, 11, 18, 21, 48]

The curves representing the total amount of phosphorus show that yeasts in the presence of amphotericin B have considerably less *P* than the normal

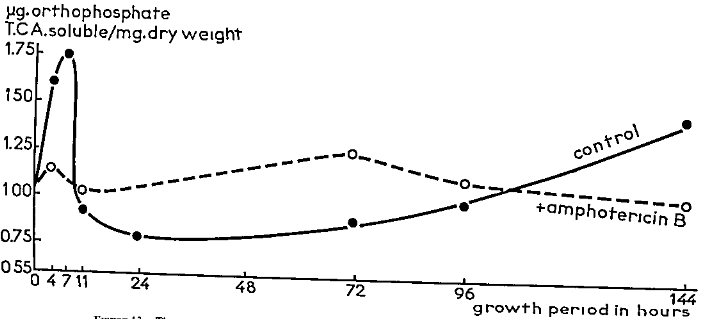


FIGURE 13. The amount of orthophosphate extracted with trichloracetic acid is diminished in treated yeasts

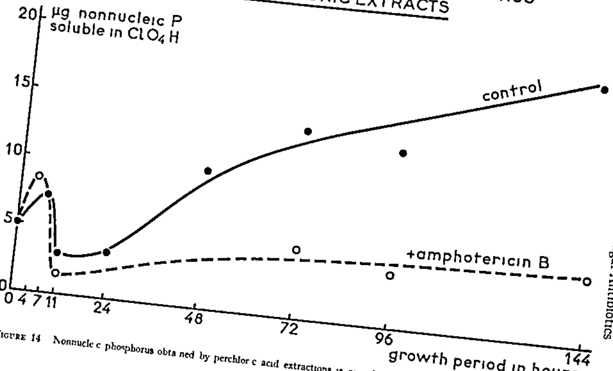


FIGURE 14 Nonnucleic phosphorus obtained by perchloric acid extractions is considerably greater in normal than in treated yeasts

cells (FIGURE 12). On the other hand, the amount of total phosphorus soluble in trichloracetic acid (TCA) is greater than the amount in normal cells. The orthophosphate of trichloracetic extracts of *C. albicans* is diminished in treated yeasts (FIGURE 13), especially the nonnucleic phosphorus of perchloric extracts, which is very abundant in normal cells (FIGURE 14). This P represents the insoluble polyphosphate of yeasts.

Therefore, amphotericin B seems to inhibit the penetration of mineral phosphorus from the culture medium or to accentuate the loss of this compound from the cells. Other studies confirmed the disorder of phosphorus metabolism of fungi treated with polyene antibiotics,[4, 40, 48] to be compared with metabolic aberrations appearing in yeasts submitted to inhibitors of the cell division.[30, 31] Osteux *et al.*[40] observed that the phosphorylated compounds marked by P^{32} are excreted by the cells submitted to the action of nystatin and that the speed of discharge is related to the concentration of the antibiotic. By utilizing chromatography and autoradiography they found that most of the total P^{32} released is in the form of orthophosphate ions. Fujii[49] found an inhibition of P^{32} incorporation on *Trichophyton gypseum* submitted to the action of trichomycin, a heptaene antibiotic. Scholz *et al.*[48] show that under the influence of nystatin on yeast there is an increase in the content of hexose phosphate. From this, the authors concluded that nystatin produced a restriction of phosphate utilization which controls physiologically the phosphorylation of reserve carbohydrates.

Possible Mode of Action of Polyene Antibiotics

The stimulation of fungal respiratory metabolism that is related to the disturbance of phosphorus metabolism under the action of polyene antibiotics supports the possibility that the mode of action of these antibiotics may be somewhat similar to that of the uncoupling agents such as dinitrophenol[35, 36, 37] and gramicidin.[16, 27] These agents may accelerate the decomposition of adenosine triphosphate (ATP) to adenosinediphosphate (ADP). This activity on ATP can explain the inhibition of the synthesis of carbohydrate reserves, of proteins, and of polyphosphates, all of which are metabolites requiring energy-rich compounds such as ATP. Under the action of polyenes, ATP may be blocked, and ADP is made available for the respiratory metabolism that is stimulated; at the same time inorganic P is released.

The exact site of action of the polyenes is yet to be determined. These antibiotics may act by stimulating the ATPase activity or, more probably, by stimulating some particular uncoupling agent naturally found in fungi, as shown recently for a mitochondrial respiration releasing factor described in animal cells. Such naturally occurring controlling factors specific for fungi may explain the activity of the polyenes only on fungi and not on bacteria, actinomycetes, viruses, or animal cells.

Summary

The action of various polyene antibiotics, particularly of amphotericin B, was studied on the metabolism of respiration, of proteins, of nucleic acids and of phosphorus compounds of *Candida albicans*.

At concentrations suppressing the growth of *C albicans*, amphotericin B produces an increase in O_2 uptake by resting or growing cells, in the presence or the absence of carbon substrate, in phosphate buffer *p*H 7 The stimulation is greater on endogenous respiration, rising to 73 per cent The stimulating effect on O_2 consumption is no longer observed on yeasts which have been washed after contact with the antibiotic, on the contrary, an inhibitory effect is seen It is possible that the washing discards the products responsible for the increase in O_2 uptake, products released by alteration of cell permeability At *p*H 4 2 the stimulation of O_2 uptake is obtained only with small concentrations of antibiotic In an atmosphere of N_2 in the absence or presence of dextrose, at *p*H 7 the CO_2 production is stimulated by the antibiotic

In the growing phase of yeasts, amphotericin B produces an inhibition of the synthesis of proteins, ribonucleic acid, carbohydrate and polyphosphate reserves, the penetration of inorganic P into the cells seems to be inhibited, but a release of P compounds is also possible The disturbance of phosphorus metabolism produced by amphotericin B is related to the stimulation of endogenous or exogenous oxidations The action of other polyenes such as amphotericin A (a tetraene), nystatin (a diene-tetraene), ascosin (a heptaene), trichomycin (a heptaene) is similar to the action of amphotericin B (a heptaene), this effect on respiration is distinct from the effect of other antifungal antibiotics such as actidione (cycloheximide) Under certain aspects, the action of polyene antibiotics is compared to the action of such uncoupling agents of phosphorylation as 2 4-dinitrophenol antifungal polyenes such as dinitrophenol, which stimulates the oxidations of animal and vegetable cells, introduced by parenteral means in man and animals, produce hyperthermia

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VIRULENCE AND GROWTH RATES OF *CRYPTOCOCCUS NEOFORMANS* IN MICE

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Previous studies of the virulence of *Cryptococcus neoformans* have investigated the relationship of encapsulation to virulence Drouhet *et al* (1950) found that a nonencapsulated variant had less virulence than the encapsulated parent yeast Kao and Schwarz (1957) studying strains of *C neoformans* isolated from pigeon nests reported no correlation between capsule size and virulence Gadebusch (1959) found that yeast cells of a nonencapsulated variant were more readily phagocytized by polymorphonuclear leukocytes from anemic mice than yeast cells of the encapsulated parent strain The studies of Littman and Tsubura (1959) indicated that an enlargement of capsule widths by cultivation of *C neoformans* on capsule agar did not increase the virulence when compared to the same strain grown on Sabouraud s agar

The results to be presented here show the range of mouse lethality for three isolates of *C neoformans* and their rates of *in vivo* multiplication

Materials and Methods

The three strains of *C neoformans* used in this study were isolated from different cases of human cryptococcosis and are designated as strains B J and S They were typical with reference to fermentation and carbon assimilation reactions did not utilize KNO_3 as a sole source of nitrogen and grew readily at 37° C Strains B and J had wide capsules and strain S had a narrow capsule *in vitro* All strains showed wide capsules *in vivo*

Suspensions of yeast cells for inoculation of mice were made from 24 to 48 hour cultures grown on 1 per cent neopeptone 2 per cent glucose agar Incubation was at 30° C Yeast cell numbers were determined by direct count in a Levy hemocytometer and diluted to contain the desired number of cells per milliliter

Female Swiss white mice weighing 17 to 20 gm and obtained from the Animal Production Section of the National Institutes of Health were used in this study

The approximate virulence for mice of each strain was determined in preliminary experiments For the mortality studies 5 serial fivefold dilutions of the initial suspensions were used Ten mice were injected intravenously with 0 2 ml of each dilution Since the strains differed in virulence the numbers of cryptococcal cells injected into the test animals varied with each strain The mice were observed for 3 months and deaths were recorded as they occurred

In vivo growth rate studies of each strain of *C neoformans* were undertaken to determine the number of yeast cells present in an organ or blood from immediately after injection and at specified intervals to and including the terminal stages of disease A standard dose of 10^5 yeast cells was injected intravenously *into each mouse and 50 to 75 mice were inoculated* with a strain

Groups of 3 mice were sacrificed at 10 min., 4 hours, 1 day, 3 days, 6 days, 10 days, 14 days, and further intervals after injection. Whole blood samples (in heparin), spleen, liver, kidney and adrenal, lung and brain from each mouse were collected separately. Each organ was weighed, ground separately in a sterile mortar and pestle, and mixed with 9 parts of sterile saline, tenfold serial dilutions were made. One tenth of 1 ml of the dilutions from each tissue suspension was placed in each of 3 Petri dishes. Molten 2 per cent glucose trypticase soy agar at a temperature of approximately 45° C. was added and agitated with a circular motion to ensure mixing of the suspension and agar. These pour plates were incubated for 48 hours at 30° C. and the colonies were counted. The lowest dilutions of those pour plates in which the colonies of *C. neoformans* varied from approximately 20 to 200, were counted. The average number of colonies on the triplicate plates was determined and, since 3 mice were autopsied, the average colony count from the organs of the 3 mice was calculated. The numbers of viable *C. neoformans* cells per 0.1 gm. of the various organs were calculated and are shown in the figures. The number of plating experiments varied with each strain. With strain B the final plating was made 16 days after injection because all the remaining experimental mice died before another plating could be done. Some of the mice injected with strain J, however, survived longer, and the final plating was made 52 days after injection of the infecting dose. With all strains the final plating experiment was made when the remaining mice were in the terminal stages of cryptococcosis.

Similar experiments, for which mice were injected intracerebrally, were conducted. For these studies each mouse was inoculated with 0.05 ml. of a suspension containing 4×10^5 *C. neoformans* yeast cells. The injections were made about one-eighth inch laterally to the midline cranial suture and about three-eighths inch above the left eye. The same plating schedule was followed as with the mice that received the intravenous inoculations but the disease progressed more rapidly under these conditions, and fewer platings could be done.

Results and Discussion

The results of mortality studies are shown in TABLE 1. It was evident that strain B had a high degree of virulence for mice considerably more so than for the other 2 strains. It must be emphasized that surviving mice were observed for a 3 month period. Most of the animals that received strain B died between 1 and 2 months following injection, whereas some of the mice that were inoculated with the higher dilutions of strain J died at a slower rate. Strain S was intermediate in its virulence.

The *in vivo* growth rate of strain B is presented in FIGURE 1. The values plotted on the graph indicate the number of viable cryptococcal cells per 0.1 gm. of tissue that were isolated at the designated time. Each point on the line represents the results obtained from a different organ by averaging colony counts on 9 plates (3 mice). Ten minutes after inoculation the lungs contained the largest number of cryptococcal cells, but viable yeast cells were found in every organ. The numbers of viable yeast cells in the lungs decreased

progressively through the third day postinoculation, but by the sixth day there was some multiplication Pour plates of the other organs showed little change in numbers of *C neoformans* cells during the first 3 plating experiments (10 min to 1 day), but an increase was apparent by the third day postinfection The numbers of cryptococcal cells in the brain increased over one thousand fold from the third to the tenth day postinoculation Other organs showed a more gradual increase, however, the spleen and the liver showed a drop on the tenth day that was not observed in cultures of the other organs After the tenth day of infection most of the organs showed a gradual increase of yeast cell numbers through the sixteenth day, but the lungs exhibited a hundredfold increase during this period It is apparent that in the terminal stages of disease the majority of viable *C neoformans* cells were found in the brain

TABLE 1
VIRULENCE OF STRAINS FOR MICE

Yeast cells injected	10^3	200	40	8	1 6
Strain B	10/10	9/10	5/10	4/10	1/10
Yeast cells injected	2×10^5	4×10^4	8×10^3	$1\ 6 \times 10^3$	330
Strain J	10/10	6/10	4/10	2/10	0/10
Yeast cells injected	5×10^5	10^5	2×10^4	4×10^3	800
Strain S	10/10	10/10	8/10	4/10	2/10

Numerator indicates the number of mice dead, denominator indicates the number of mice injected All mice were injected intravenously

FIGURE 2 shows the growth rate of strain J in mice The results obtained from the plating experiments through the tenth day postinfection were similar in most respects to those from strain B After this point, however, there was a significant change The numbers of viable cryptococcal cells cultured from the mice 14 to 44 days postinoculation remained about the same There were considerable variations in some instances, but the growth curve during this period was relatively level The results of the final plating experiment indicated an increase of yeast cell numbers over the previous plating but the level in the brain was not as high as with strain B

The multiplication of strain S *in vivo* is shown in FIGURE 3 The results obtained with this strain for the first 6 plating experiments (10 min to 10 days) were quite similar to those obtained with the other 2 strains The greatest difference when compared to the other 2 strains is the larger number of viable yeast cells that were isolated from the kidneys for the last 4 plating experiments

The *in vivo* multiplication of strain S following intracerebral inoculation is presented in FIGURE 4 *All 3 strains were studied in this manner Since* strains B and J gave similar results, only the results obtai strain S are shown The mice inoculated by this route received ap $\times 10^4$

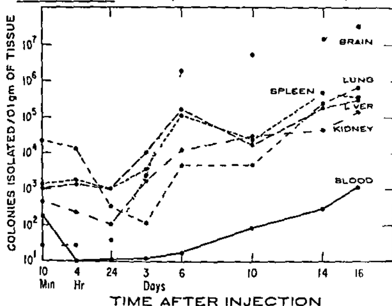


Figure 1 Multiplication of strain B in mice following intravenous inoculation with 10^5 yeast cells.

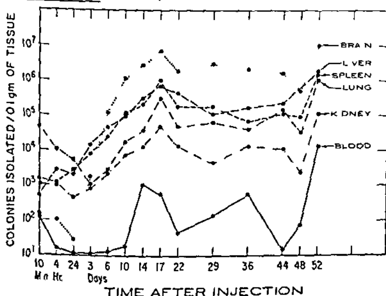


Figure 2 Multiplication of strain J in mice following intravenous inoculation with 10^5 yeast cells

C neoformans, STRAIN S, INTRAVENOUS INOCULATION 10^5 CELLS

COLONIES ISOLATED /0.1 gm OF TISSUE

BRAIN
KIDNEY
LUNG
LIVER
SPLEEN
BLOOD

10^1 10^2 10^3 10^4 10^5 10^6 10^7

10 Min. 4 Hr 24 3 Days 6 10 14 17 21 25 32

TIME AFTER INJECTION

FIGURE 3 Multiplication of strain S in mice following intravenous inoculation with 10^5 yeast cells

C. neoformans, STRAIN S, INTRACEREBRAL INOCULATION, 2×10^4 CELLS

COLONIES ISOLATED /0.1 gm OF TISSUE

BRAIN
LUNG
LIVER
SPLEEN
KIDNEY
BLOOD

10^1 10^2 10^3 10^4 10^5 10^6 10^7

10 Min. 4 Hr 24 3 Days 6 10 14 17

TIME AFTER INJECTION

FIGURE 4 Multiplication of strain S in mice following intracerebral inoculation with 2 yeast cells

cells As expected, the brain had more viable yeast cells 10 min after injection than the brains of mice that received intravenous inoculations but the lungs still showed the greatest number per unit weight Otherwise the 3 initial plating experiments gave similar results when compared to those of mice receiving intravenous injections Growth of *C neoformans* was more rapid and the greatest rate of multiplication occurred in the brain from 3 to 6 days after injection The large numbers of cells isolated from kidney tissue following intravenous inoculation with this strain were not observed in this experiment The reduced experimental time may have been an important factor in this observation

Examination of the results of these experiments indicated a number of factors that merit discussion It was quite apparent that most of the yeast cells were removed from the circulating blood quite rapidly following [illegible] and tended to accumulate in the lungs This was demonstr[illegible] inoculated either intravenously or intracerebrally and most probabl[illegible] was a mechanical phenomenon The cells of *C neoformans*, being qu[illegible]rge possibly were trapped in the pulmonary capillaries The gradual [illegible] of viable yeast cells in the lungs suggested that they were either [illegible] out subsequently or that they were destroyed by the host The smaller numbers of viable cryptococcal cells were isolated 4 to 24 hours after injection and this observation supports the contention that the host defenses were able to destroy some of the yeast cells

Multiplication *in vivo* of all the strains injected intravenously during the first three days was generally the same Three days after infection however strain B multiplied quite rapidly, and the mice were dead or dying [illegible] in TABLE 1, which indicated a high level of virulence for this strain Strain J which possessed considerable virulence according to TABLE 1 was less virulent than strain B, as indicated by FIGURE 2 The most interesting result obtained with that strain was the relatively constant numbers of viable yeast cells that existed in the organs of the infected mice from the fourteenth to the forty fourth day postinfection While this appeared to be a more compatible host parasite relationship than existed with the other strains eventually the host resistance broke down, and further multiplication was observed The number of yeast cells in the brain, however, was not as high as with strain B but was comparable to the levels observed in mice that received strain S

Mice were considerably more susceptible to intracerebral inoculation of *C neoformans* than to intravenous injection An inoculum one fifth that used for intravenous studies produced terminal cryptococcosis in about one half the time

The results that have been presented were obtained from surviving mice that had been sacrificed at the specified time intervals and do not account for animals that died of cryptococcosis during the experimental period While the mice that were autopsied were not selected with regard to overt cryptococcosis, the methods employed measure the growth rate in the more resistant animals, particularly in the experiments that were extended over longer periods of time This must be considered especially in the interpretation of the data

obtained from the intravenous inoculation with strain J and, to a lesser extent with strain S

These results indicated that all of the strains utilized in this study demonstrated considerable virulence for mice, but there were variations in respect to the rates of multiplication and the total numbers of *C neoformans* cells isolated

Summary

Mortality studies in mice with three strains of *C neoformans* revealed a considerable variation in virulence that was not correlated with capsule width

In vivo growth rates of the same strains, obtained by making colony counts from pour plates made from blood ground spleen, liver, kidney lungs and brains from intravenously infected mice autopsied at specified time intervals indicated similar rates of multiplication during the first 3 days Thereafter 1 strain grew rapidly, and the test animals were in the terminal stages of disease 16 days postinoculation The other 2 strains produced similar stages of disease in surviving mice 32 and 52 days after injection Immediately after inoculation the lungs contained the largest numbers of yeast cells The colony count decreased 1 to 1 5 logs on days 1 to 3 and subsequently reached a level of approximately 10^6/0 1 gm lung tissue In terminal cryptococcosis the largest numbers were isolated from the brain

C neoformans inoculated intracerebrally showed a faster rate of multiplication than when injected intravenously, and the mice were moribund sooner even though a smaller dose was given

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THE ACCURACY OF SEROLOGIC METHODS IN DIAGNOSIS

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Serologic tests have been employed increasingly during the past dec[illegible] the diagnosis and study of coccidioidomycosis histoplasmosis and [illegible]mycosis These tests have played a prominent role in the detection of less new cases, especially of coccidioidomycosis and histoplasmosis and [illegible]se of this, their contribution to the increased over all knowledge of these inf[illegible]ons has been considerable The alleged shortcomings, of which there are [illegible] of these tests are due in part to the crudeness of the antigens rather th[illegible] tests per se, but also to the failure of many persons to recognize the [illegible] infections as dynamic diseases That antibody response will vary [illegible] type, severity, duration and, particularly, with the activity of a my[illegible] infec[illegible]tion, as it does in an infection produced by any other microorganism [illegible] been especially difficult to convey

Certainly, too, the earlier claims of even a qualified specificity for [illegible] the antigens were unwarranted[1-5] There are strong cross reactions [illegible] sera from culturally verified cases of histoplasmosis and coccidioidomycosis [illegible]tomyces antigens, and in those of blastomycosis and coccidioidomycosis with histoplasma antigens Even with coccidioidin, the most specific of th[illegible] commonly employed, there are reactions in sera from frank cases of histoplasmosis sometimes to significant titer[6] Moreover there is little reason to doubt that some of these antigens also react with sera from others of the systemic mycotic infections that have not yet been studied serologically principally cryptococcosis[7]

To assess the accuracy of serologic diagnosis one must consider also not only the specificity of an antigen, but the type and stage of an infection from which a serum is taken, as well as certain natural sequelae that obtain thereafter If it can be assumed that antibodies are stimulated in direct proportion to the number of accessible organisms, one would expect the concentration of antibody to be minimal, and perhaps not even of a demonstrable level in an asymptomatic individual with a single, pulmonary lesion, just as one would expect concentration of antibody to be high in patients with extensive pulmonary infiltration In addition, there is the matter of viability of the organism and the host s ability to eradicate it When microorganisms are phagocytized by mobile defense cells, they probably not only are rendered nonviable but are rapidly eliminated from the host, and a rapid loss of antibody production following removal of the stimulus ensues There are other organisms, however, that escape the mobile agents of defense and become 'trapped" by the stationary cells they have parasitized Here, in protest, the host's calcific, granulomatous, or fibrotic tissues are produced, in which the organisms usually meet a slow disintegrating death However, as long as the organisms or fragments of the organisms are present in these tissues, even though they are nonviable and culturally impossible to recover,[8-10] it is possible that they continue to produce antibodies to some of the several antigenic components they contain These residual anti

bodies may persist for very long periods, depending upon the extent and site of the lesion, the state of the patient's immune mechanism, and a host of other factors. Such residuals are an inherent part of the immunological process and their production and tenure always must be regarded in the assessment of the activity and specificity of an antigen. Because of the widespread and as yet not completely defined geographic distribution of some of the mycotic agents,[11,12] there is reason to believe that the serologic titers observed in many apparently healthy persons are residuals from earlier unrecognized infections caused by mycotic agents rather than nonspecific reactions of the antigens as is so often implied.

With these elemental considerations in mind, the serologic findings in coccidioidomycosis, histoplasmosis, and blastomycosis, the problem of cross reaction and some of the factors involved in the development of improved antigens are reviewed and discussed.

Materials and Methods

The materials, methods, and procedures most widely employed in the routine serologic diagnosis of coccidioidomycosis, histoplasmosis, and blastomycosis are described in numerous earlier reports.[1,6,13,22]

Complement fixation (CF) is the serologic procedure most universally used generally with four antigens in simultaneous tests. These antigens are:

(1) *Coccidioidin.* An asparagine-broth filtrate in which strains of *Coccidioides immitis* are grown in the filamentous phase for periods of several months.[1] This antigen is referred to as C in this report.

(2) *Histoplasmin.* A similar preparation from strains of *Histoplasma capsulatum* in the filamentous phase.[23,24] This is the antigen referred to as D in this report.

(3) *Histoplasma whole yeast phase antigen.* A standardized suspension of killed 48- to 72-hour-old whole yeast cells of *H. capsulatum* in buffered saline solution, prepared from single or multiple strains.[19,22] This type of preparation is referred to as the H antigen in this report.

(4) *Blastomyces antigen.* A saline extract of mechanically disrupted yeast cells of *Blastomyces dermatitidis* prepared from single or multiple strains.[6] This antigen is the one referred to as B in this report.

Precipitin tests are also used routinely in suspected coccidioidomycosis[1] and by some laboratories in suspected histoplasmosis.[20] The filtrate antigens coccidioidin (C) and histoplasmin (D) are employed.

Collodion agglutination is a third procedure used in several laboratories in the study of histoplasmosis.[18] Histoplasmin (D) is the antigen utilized.

Serologic Reactions in Coccidioidomycosis

In many cases of mild to moderately severe primary, pulmonary coccidioidomycosis, CF antibodies are never demonstrable. This is in contrast to precipitins that, as shown in FIGURE 1, can be demonstrated in 90 per cent of the patients with this type of infection during the second to fourth week of illness. Precipitins however are transitory. They no longer are demonstrable in approximately 50 per cent of the primary nondisseminating cases by the fifth to seventh

week of disease The establishment of diagnosis by serologic methods in this type of case, therefore, is especially dependent upon the time after onset that sera are tested

In those primary nondisseminating cases in which CF antibodies are produced, demonstrable levels usually remain low (1 2 to 1:16), are much later in developing, and persist for much longer periods than the precipitins In the series of cases shown in FIGURE 1, it will be noted that less than 10 per cent of the patients were CF positive during the first week of illness in contrast to the 50 per cent with positive precipitin tests During the twenty fourth week, however, 90 per cent of the 49 persons remaining under observation in this

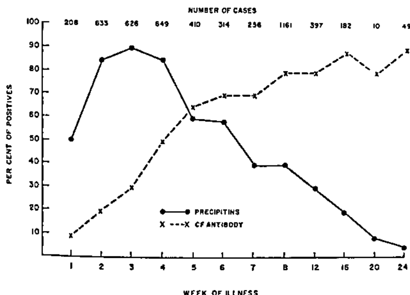


FIGURE 1 Precipitins and CF antibodies in sera of patients with primary nondisseminating coccidioidomycosis [3]

particular study still had demonstrable CF antibodies, whereas approximately 95 per cent no longer had precipitins [3]

Although the CF test is of limited value in the diagnosis of many mild to moderately severe primary cases, it is invaluable in the prognostic evaluation of infections that are more severe A CF level of 1:32 or above is generally regarded as an indication of dissemination, with the magnitude of the titer indicating the amount of extrapulmonary tissue involved Persisting high CF levels of 1 64 to 1:256, or progressively increasing titers of 1 32 to 1 128–512, are indicative of progressively disseminating disease, while progressively diminishing levels of CF antibody, despite former higher ones, are generally associated with clinical improvement [3]

As shown in FIGURE 2, however, changes in a patient's clinical status and

serologic pattern may occur within weeks or not for months or years. Curve A is illustrative of the CF levels observed in progressively disseminating cases and generally occurs within weeks or months, but may occasionally take years. The CF levels in curve B are seen in "stabilized" disseminated infections of long duration. This pattern generally foreshadows further dissemination although patients may occasionally recover. The decreasing CF titers in curve C are observed in patients showing progressive clinical improvement in spite of earlier dissemination. Curve D is of particular importance as it is the notable exception to the association of low CF titer and good prognosis. While it is true that low levels of this order are seen in many patients with pulmonary cavities or single extrapulmonary lesions for whom prognosis is

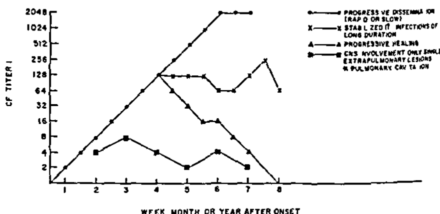


FIGURE 2. CF antibody patterns in disseminated (extrapulmonary) coccidioidomycosis.

fair or good this pattern is also observed in patients in whom the solitary lesion is located in a vital meningeal area. In the usually fatal meningeal infections with no other tissue involvement, the low CF levels thus fail to support the grave prognosis.[2] In the serologic study of coccidioidomycosis there is another little mentioned element of danger. Residual CF titers of 1:2 to 1:8 may persist in some persons for many years following recovery from a disseminated infection. This possibility of residual coccidioidomycosis is perhaps too seldom considered in the differential diagnosis of a more recently acquired upper respiratory illness that is of bacterial or viral origin, especially in persons removed from the endemic areas for coccidioidomycosis.

Serologic Reactions in Histoplasmosis

The serologic findings in histoplasmosis are not as well classified or as meaningful as they are in coccidioidomycosis in spite of, or perhaps because of the more widespread geographic distribution of *H. capsulatum*.[11,12] In coccidioidomycosis the serologic data were comparatively rapidly accumulated in the comprehensive study of thousands of service personnel newly introduced into the endemic areas during the years of World War II. These studies moreover

were carried out by a single team of imaginative and energetic inve tigators who employed a single lot of coccidioidin in two unvarying serologi pro cedures[3] In contrast the serologic findings in histoplasmosis have been p ed together from fragments of information obtained by a number of diff r t investigators from sporadic cases or sporadic small series of cases in d separated geographic regions To a large extent the antigens were pr par l by the individual investigators and thus varied not only from lot to lo l even as to the biphasic morphology of the organism[1 2] Because no one t of ant gen proved to be reactive in all types of cases antigens from both gr th

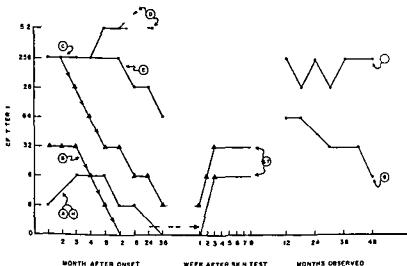


FIGURE 3 CF ant body patterns in histoplasmos s B m ld primary pulmonary D fatal infan le type or rap dly d ssem nat ng C severe pr mary pulmonary F chron c pulmonary F large cavity (single or mult ple) G small cavity (s ngle or mult ple) A & H sol tary pulmonary nodule d sseminated with lymphadenopathy and ST sk n test

phases of *H capsulatum* have been used simultaneously in all routine CF tests for the past several years

CF is the one serologic procedure employed universally in the study of histoplasmosis In some laboratories this is augmented by the precipitin test[20] and in others by the collod on agglutination test[18] Both of the latter assist primarily in the early detection of mild primary pulmonary cases for as in coccidioidomycosis CF antibodies are never demonstrable in some of the milder primary infections caused by *H capsulatum*[4 15 20]

The recognized serologic patterns in histoplasmosis moreover are not transitional from one type of the infection to another as they appear to be in dissem nated and nondisseminated cases of coccidioidomyco is Instead they are merely a series of patterns that have been observed repeatedly in certain types of culturally verified but unrelated cases[4 19] As illustrated by

curves C, D, E, F and, possibly, G in FIGURE 3, some of these patterns have attained diagnostic stature even when attempts to isolate the organism meet with failure At this point, it is pertinent to note that *H capsulatum* is perhaps the most difficult of all the pathogenic mycotic agents to recover by cultural procedures, and is especially difficult in the primary pulmonary case In this regard, it is fortunate that the severe to moderately severe primary infection as shown in curve C, FIGURE 3, elicits a high CF antibody response during the first month of illness In uncomplicated cases, the elevated titers persist for several additional weeks or months and then rapidly decrease to residual levels of 1 8 or 1 16 These residual levels may remain unchanged for from 3 to 5 years, or the patient's titer may become negative in as little as 6 months In either instance, the patient has apparently completely recovered clinically within 3 to 5 months after onset In still a third type of apparently uncomplicated primary case, the elevated CF levels shown in curve E persist for as long as a year, although clinical recovery also appears to be complete after only a few weeks or months The first portion of curves C and E is often indistinguishable from that of the CF patterns seen in fulminating and usually fatal infections in infants or young children and, occasionally, in adults whose infections are complicated by one of the prognostically grave blood dyscrasias In these more severe cases, however, CF titers rarely decrease, but remain high even in sera collected at autopsy (curve D) Curve F is characteristic of the serologic pattern of persons in whom single or multiple large pulmonary cavities remain unchanged throughout several years of observation In these cases, very high CF levels persist, with little variation over a period of years By contrast, when cavities are small there is much less serologic response, as shown by curve G The CF titers usually are not remarkably high and tend to diminish when the patient is treated by bed rest alone For this reason, the patient with small cavities is easily overlooked if serologic results are the sole criteria for excluding the infection CF titers observed in curves C, D, E, and F, however, are generally regarded as diagnostic

On the other hand, curves AH, B, and ST have little, if any, diagnostic significance, except perhaps to increase the physician's index of suspicion in diagnostically puzzling cases Curve AH is representative of the low CF titers and, in some cases, even these are absent, as seen in patients with the single pulmonary nodule that requires differentiation from carcinoma In this very common type of the infection (histoplasmoma), serologic tests have no diagnostic value The concentration of CF antibody because of the limited amount of tissue involved does not exceed that carried by a substantial percentage of the normal population Paradoxically, curve AH is also representative of the highest serologic response demonstrable in the most severely disseminated and usually fatal type of histoplasmosis that often is concurrent with Hodgkin's Disease, Addison's Disease, or sarcoidosis[6,19] In most of these cases serologic reactions are negative for months prior to death although at autopsy the major organs are teeming with *H capsulatum* Curve B is seen in the milder cases of primary, pulmonary histoplasmosis but unless it is observed in an epidemic in which there are more severe infections it has doubtful diagnostic significance The magnitude and duration of CF titers in such

cases are remarkably similar not only to those observed in culturally r ed cavitary cases (curve G) but also to those in previously sen itized or r fe l persons (curve ST) whose antibody production has been timulat l b recent application of the histoplasmin skin test [25]

In brief CF titers of 1 8 or 1 16 with histoplasma antigen are t l be used as diagnostic criteria A recent summary of serologic findi g 6 persons hospitalized with chest diseases revealed that titers f 1 S l were observed in approximately 80 per cent In contrast ser l reached or exceeded 1 32 in 75 per cent of the 52 culturally verific l n this study The verified cases that did not react at the 1 32 level v al healed minimal and generally inactive infections [26] To e tabl l presumptive diagnosis of active histoplasmosis on CF titer of le h therefore appears to be an extremely tenuous procedure

Serologic Reactions in Blastomycosis

In our experience, the serologic diagnosis of blastomycosi i couraging than that of histoplasmosis With the antigen empl laboratory and sera from 107 culturally verified cases th se fr 68 per cent) proved to be serologically negative On the other han obtained with this same antigen have equaled or exceeded tho the homologous ones in a high percentage of the culturally verified plasmosis and coccidioidomycosis coming to our attention during 10 years [6] This antigen not only is one of the least specific of the 4 rr ntly employed in routine tests but it is al o strikingly unreactive r fr m many culturally proven cases of blastomycosis A CF reaction to bla ton antigen may be helpful in evaluating the prognosis of a verified e[6] but th the type of antigenic preparation we employ CF reactions merit no pla e in the diagnosis of this disease as further demonstrated below

The Problem of Cross Reactions

In some of the earlier reports, qualified claims of specificity were ma le f r each of the antigens used in serologic as well as dermal sen itivity tests [21] When with further use, numerous strong cross reactions were observed it was maintained that the commonly shared or cross reactive components could be eliminated or diminished by appropriate dilution [24]

The antigens used at the Walter Reed Army Institute of Research for the serologic study of mycotic diseases are critically standardized in sera from a variety of types of histoplasmosis blastomycosis and coccidioidomycosis in humans Moreover, because the patients involved usually are military personnel many of whom have lived in each of the areas known to be most highly endemic for these three mycotic diseases each serum submitted is tested simultaneously with antigens of *H capsulatum B dermatitidis* and *C immitis* in the CF test Collodion agglutination with histoplasmin and precipitin tests with coccidioidin also are done The results of CF tests on more than 50 000 sera reveal that cross reactions are extensive In precipitin and collodion agglutination tests cross reactions are even more confusing

Although some of these findings were discussed in considerable detail in an

earlier report,[6] the magnitude of the problem with the CF test antigens alone is illustrated further in FIGURE 4 Of the 10,000 serum specimens most recently received in this laboratory, 6759 were CF negative with all 4 of the antigens used, and 602 were anticomplementary A total of 2639 had CF titers of 1 8 or above with one or more of the antigens Single reactions were observed in 684, 389, 293, and 193 specimens with the D, B, H, and C antigens, respectively, and a total of 231 additional specimens reacted only with the 2 types of histo-

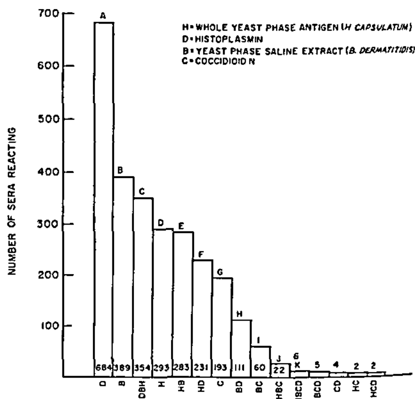


FIGURE 4 Distribution of cross reactions with mycotic antigens in 2639 consecutive specimens submitted for routine serology

plasma antigen In the remaining 849 sera, however, strong cross reactions were present There were 354 that reacted to essentially equal CF titers with antigens H, B and D (Column C), 283 with antigens H and B (Column E) 111 with B and D (Column H), 60 with B and C (Column I), 22 with H B and C (Column J), and 6 with all 4 of the antigens, H, B, C, and D (Column K) Other sporadically observed combinations are also illustrated

The Transitory and Variable Nature of Cross Reactions

Cross reactions are most extensive in sera from primary, pulmonary cases of both histoplasmosis and coccidioidomycosis, in which it is not unusual for CF

titers with blastomyces antigens to exceed the levels of those with either of the homologous ones. This pattern is never seriously suggestive of blastomycosis, however, as the acute, febrile, respiratory illnesses are more common in association with infections caused by *C. immitis* or *H. capsulatum*. On the other hand, this particular cross reaction poses a serious differential problem between coccidioidomycosis and histoplasmosis, especially if the serum is drawn from a patient with a mild case of coccidioidomycosis in which CF antibodies for coccidioidin are never demonstrable. Unfortunately, it is not uncommon for both histoplasma and blastomyces antigens to react to CF titers as high as 1:64 in coccidioidin CF negative sera from patients with primary coccidioidomycosis. Coccidioidin CF titers, conversely, are observed in the sera of patients with histoplasmosis only when the homologous CF titers are very high (1:512), and then only at levels of 1:8 to 1:32.[6] A cross reaction that is even more difficult to evaluate occurs in pulmonary cavitary cases of histoplasmosis, blastomycosis, and coccidioidomycosis. Histoplasma and blastomyces antigens react to high and equal CF levels in many of these cases regardless of the etiological agent, while CF titers with coccidioidin are low or absent even in the cavitary disease produced by *C. immitis*.[3,6,27,28] In primary pulmonary cases increasing titers with homologous antigens, accompanied by diminishing levels with heterologous ones in subsequent serial serum specimens, frequently help resolve the diagnosis, and this is one of the reasons serial specimens over an extended period are requested. In the cavitary and more chronic type of mycotic infection in which strong crosses occur, however, the confusing serologic pattern may remain unchanged for months or even years.[26]

These findings leave little doubt that there are antigenic components commonly shared by these three and possibly other systemic mycotic agents. It is inconceivable that there are this many dual infections. The transitory and variable nature of the cross reactions, moreover, suggests that different types and concentrations of antibodies are produced by the various antigenic components of these agents at different stages of an infection.

A hypothetical schema, attempting to illustrate the dynamic and progressive processes involved in the production of antibodies at different stages of an infection to a number of antigenic components, some of which are shared by other organisms, is presented in FIGURE 5. In the configurations, also hypothetical, of the crude antigens of *H. capsulatum*, *B. dermatitidis*, and *C. immitis*, it will be noted that the *B. dermatitidis* antigen contains three times as much component A as coccidioidin and almost twice as much as the histoplasma antigen. Let it be assumed that the serum to be tested for CF antibody is from an active case of histoplasmosis early after onset. The antibody composition of this serum is a constant just as the higher concentration of component A in the blastomyces antigen is a constant. Therefore, if at the time the serum is drawn the patient has produced only a minimal amount of antibody to component A and none to the other components of *H. capsulatum*, this serum will react to higher titer with the blastomyces antigen than with the homologous one. However, if in later specimens the concentration of antibody to fraction A increases, the CF titer with the histoplasma antigen will more nearly approach the level of the blastomyces antigen. When anti-

body to A is produced in very high concentration, even the small quantity of fraction A in coccidioidin will react, but always at lower levels than the other two antigens. Attempts to eliminate this cross reaction with blastomyces antigen either by dilution of the antigen or by adsorption of the serum will lead only to earlier negative reactions with histoplasma antigen and coccidioidin, as these contain the lesser concentrations of component A.

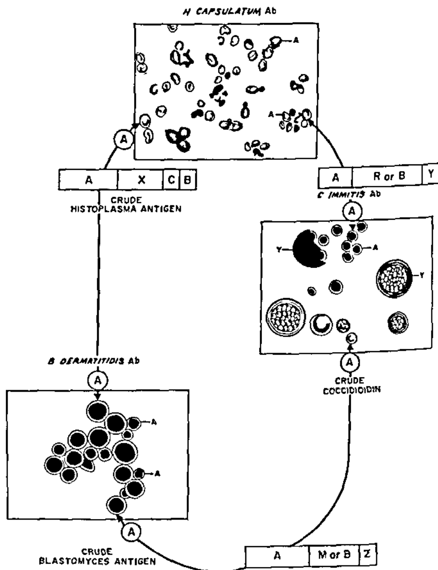


FIGURE 5. Schema (theoretical) illustrating basis for cross reactions in sera from culturally verified cases of histoplasmosis, blastomycosis, and coccidioidomycosis.

In an infection that is active and of sufficiently long duration antibodies to the other components of *H capsulatum* in time also will be produced by the patient, while antibodies to A may or may not diminish In any event antibodies to these other fractions will then react with the components of *H capsulatum* which neither *B dermatitidis* nor *C immitis* possesses and titers with the histoplasma antigen may begin to exceed those with the blastomyces antigen However, the blastomyces antigen will continue to react as long as antibodies to component A persist, and to higher titer than the histoplasma antigen as long as production of antibody to component A exceeds the production of antibody to any of *H capsulatum's* other components

If components in addition to A are shared commonly by these agents the problem becomes increasingly complex For example component R in *C immitis* might also be related closely to component M in *B dermatitidis* and both of these in turn might be essentially the same as component B in *H capsulatum* Antibodies to this component might also be produced at different stages of these three as well as other mycotic infections

The development of different types of antibody at different stages of an infection is perhaps illustrated more comprehensibly in *C immitis* infections because of the varying morphology of this organism *in situ* For example let it be assumed that the chemical composition of the walls of endospores (component A, FIGURE 5) differs from that of the walls of mature spherules (component Y, FIGURE 5) due to lengthened or more complex polysaccharide moieties in the latter[27,29] In the patient in whom few endospores have yet developed into mature spherules the antibody produced by component A could be expected to predominate and, at certain early stages of the infection be the only one demonstrable, in a second patient in whom there is a continuous cycle of maturation of spherules and further release of new endospores antibodies might be developed to both components A and Y and, in a third patient whose infection has stabilized with innumerable mature spherules and no further release of endospores, antibody could be expected to be produced primarily to component Y Provided component A is the one shared by both *B dermatitidis* and *H capsulatum*, the latter antigens would react to equal or higher CF titers than coccidioidin in early mild to severe primary nondisseminating and in rapidly disseminating infections of coccidioidomycosis In disseminated infections, or in those in the process of slow dissemination, antibody to component Y would supersede antibody to component A Eventually, therefore, coccidioidin the only one of these three antigenic complexes that contains the Y component would also be the only antigen that would react with sera from the cases of coccidioidomycosis of longer duration

As noted earlier something of this nature does appear to occur in naturally acquired coccidioidomycosis Histoplasma and blastomyces antigens react to higher CF titers in early sera from primary pulmonary cases in which coccidioidin CF titers are low or are frequently absent, to essentially equal CF titers with coccidioidin in rapidly disseminating infections and not at all in moderately severe to severe disseminated and nondisseminated cases of longer duration[6]

In histoplasmosis, there is also considerable evidence that the antibody

produced in early infections differs from that demonstrable in chronic or older cases. As described in an earlier report, high CF titers with the whole yeast phase antigen of *H. capsulatum* were observed in sera from primary, pulmonary cases in which histoplasmin failed to react, whereas in chronic infections of longer duration the high CF titers were obtained with histoplasmin in contrast to negative reactions with the whole yeast phase antigen. There were also sera from certain cases, as might be anticipated, which reacted with both types of antigen to essentially equal CF titers.[30] This difference in reaction with the two types of histoplasma antigen is illustrated further in FIGURE 4 where more than twice as many specimens reacted with histoplasmin (D) as with the whole yeast phase antigen (H), and an additional 231 specimens reacted with both antigens (H and D) to essentially equal CF levels. There is little reason to doubt, moreover, that the same variation in antibody concentration to various antigenic components of *B. dermatitidis* does not also occur in blastomycosis, or that certain of the components of *B. dermatitidis* do not elicit antibody at certain stage(s) of this infection that are as readily demonstrable by histoplasmin as by blastomyces antigens. As in histoplasmosis and coccidioidomycosis, there are also cases of blastomycosis in which cross reactions do not occur, although the same antigens are employed.

Discussion

The accuracy of serologic diagnosis in these three mycotic diseases is thus difficult to assess as it depends not only on the composition of an antigen but on the type, severity and duration of infections from which sera are taken. In the assessment of any biological procedure it is important to know what factor one is measuring and in the serology of mycotic diseases, there are still countless unknown entities.

The crude antigens currently employed have been used to good advantage in many ways, but further progress can not be expected from their continued use in their present state. Not only is the antigenic composition of such preparations unknown, but the concentration of the various components varies from lot to lot and, possibly from strain to strain. Moreover, there is little evidence to suggest that the antibodies with which they react are protective, as CF titers frequently increase with dissemination and decrease with clinical improvement. The protective antigenic component either is not present or is overshadowed by higher concentrations of other less important, and perhaps cross reactive, components. In addition, there doubtlessly are other components associated with residual antibody from past infections, or involved in the stimulation of antibody following skin tests that possibly could be differentiated from those of importance in the more active stages of these infections.

All pertinent serologic findings up to the present time indicate that the various antigenic components and the antibodies they produce can not be resolved independently of each other if serologic analysis is to contribute further to the knowledge of the mycotic infections. Efforts to develop crude antigens containing a maximum number of components for fractionation studies perhaps would represent the first step in this direction, as components can not be isolated from preparations in which they do not exist. Even in

the same type of preparation, the number and concentration of comp[illegible] vary For example, in four lots of histoplasmin simultaneously te[illegible] agar gel diffusion in this laboratory, the number of reactive comp[illegible] varied from one to six with the same serum Evidence that they vary [illegible] more with change in nutritional factors and time and temperature of incul[illegible] of the etiologic agent is provided by the dissimilar reactions observed [illegible] crude antigens prepared from yeast and filamentous growth phase [illegible] *capsulatum* (FIGURE 4)

On the other hand, the possibility that different antibodies are prod[illegible] different antigenic components at different stages of an infection sh[illegible] be overlooked Heiner, using a single lot of histoplasmin in an agar [illegible] fusion technique, demonstrated that the number of reaction bar[illegible] vari[illegible] sera from individual patients as well as in those from persons w[illegible] histoplasmin skin tests[30]

It is possible, of course, that the variable and transitory nature [illegible] and cross reactions in mycotic diseases might be explained on th[illegible] inhibitory substances, antigen excess or immunological paralysis in th[illegible] but there are many instances in which none of the long accepted exp[illegible] for serologic failures seem to apply It is perhaps pertinent to [illegible] that many of the current concepts of antigen antibody reactions wer[illegible] from studies with bacterial agents that reproduce so rapidly that antigen[illegible] flagellar, somatic, or capsular constituents appear to arise simultaneously Because of the rapid overlapping of generations antibodies to one constituent would appear to develop as rapidly as those to any other The rate of growth in pathogenic mycotic agents, by contrast, is so extremely slow that antibodies elicited by an agent's antigenic complex at one stage of its development might be readily distinguished from those developed later in an infection to the more complex array of chemical moieties in the mature organism This difference in the rate of growth might be of importance in the immunological process of mycotic diseases and appears to merit further consideration

Accurate serologic analysis, therefore, can not be achieved except by the isolation and characterization of each antigenic component an organism produces, related as well as unrelated, and the further correlation of these components' activities with antibodies produced throughout all stages of an infection In the serologic study of mycotic infections it is hoped that the cross reactive components can be deleted in order that those involved in the production of protective or residual antibody, or both, can be more carefully evaluated

The resolution of this formidable, challenging problem will require the collaborative efforts of immunochemists pathologists serologists mycologists, and clinicians However, we are not alone in this dilemma In a recent study of the venerable tubercle bacillus reported by Williams and Dubos[31] the following statements were made It must be remembered on the other hand, that the whole tubercle bacillus is a rich packet of biological activities all theoretically residing in substances capable of chemical definition Combined in fractions or associated in complex in their natural state, or recombined in test tubes, it is reasonable to suppose that the activities of such substances,

revealed by suitable experiments in suitable animals, could be complementary, supplementary, masked, antagonistic, inhibitory, or combinations of these It remains to be seen whether the changing morphology of the pathogenic fungi renders the mycologist's problem more or less complex than that of the bacteriologist

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THE QUESTION OF IMMUNITY IN RINGWORM INFECTIONS*

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There is some indication that an immune response against dermatophytes is developed in experimental animals in view of the early work of Delamater[1] and Delamater and Benham[2] with *Trichophyton mentagrophytes* and of the more recent work of Reiss and Leonard[3] with *Microsporum canis* and of Keeney and Huppert[4] with *T mentagrophytes* Also Huppert and Keeney[5] felt that they had demonstrated an acquired resistance against dermatophyte infections in human volunteers previously immunized with antigens of *T mentagrophytes* For the most part however the immunity developed has been partial in that the course of the disease following immunization was only shorter and milder than that normally observed The role of such an altered response under natural conditions is nebulous in part because of so called natural resistance to dermatophyte infections that has been described by many particularly well by Barlow[6] It is generally assumed that most persons are exposed to dermatophyte infections yet relatively few develop clinical disease This is well exemplified by the study of Kligman[7] who found that only 5 per cent of a group of institutionalized children had acquired a natural infection during an epidemic of tinea capitis caused by *Microsporon audouini* despite the intimate and prolonged contact this situation provided The factors that limit the spread of such epidemics would seem to be more complex than merely the natural resistance of certain individuals and might well be strongly influenced by other factors such as infectivity and virulence of the strain size of the inoculum received by the individual and the effect of local trauma as well as the possibility of altered response following infection For example it is our impression based more on speculation than data that *M audouini* although perhaps more virulent than other of the causative agents of tinea capitis may not be as infectious It was our desire to determine the presence or absence of immunity under natural conditions following recovery from naturally acquired tinea capitis The observation that children usually do not have second bouts with this disease would seem at first glance sufficient evidence for a concept of acquired immunity However, since the opportunity for first infections often is not great the failure to acquire a second infection might well be attributable to lack of opportunity rather than immunity Of 718 children treated for tinea capitis in the Tulane University clinic during a three year period not one returned with a second infection despite entirely free medical care including medication

At the time griseofulvin first became available a deliberate effort was made to set up studies to explore the possibility of immunity following infection It seemed to us that treating one sibling and then leaving that child exposed to one or more untreated children would provide a long awaited opportunity

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This we d d and are in the process of continuing Thus far 15 of tinea capiti patients treated with griseofulvin have not become reinfected after known exposure An interpretation of these results was not possible however unless we first determined the rate of infectivity of normal individuals under similar exposures It seemed to us that we should ascertain those condition that would provide maximum exposure and infectivity for normal individuals then if our previously infected individuals did not become infected under such conditions there might be some grounds for the concept of acquired immunity To do this a study was made of 460 *M audouini* infections among 2o7 Negr families Three hundred and fifty-one of the total infections occurred i males 331 of whom had tinea capitis and 20 claimed to have had only tin a corporis This ratio was only slightly higher than the incidence usu lly

TABLE 1
FAMILIES WITH SINGLE CASES OF NONINFLAMMATORY TINEA CAPITIS CAU ED BY *MICROSPORUM AUDOUINI* WHO EXERCISED NO PRECAUTION TO PREVENT INFECTION OF SUSCEPTIBLE MALES

Infected males		Non nfected suscept ble males	
Age	Duration of fluorescence (mos.)	No	Ages
6	2	1	9†
8	12	6	11† 11† 10† 9 3 6
10	6	2	9† 7
9*	7	1	8†
7	9	3	13† 11 2
8*	3	3	5† 4
7	7	2	5† 12
8	9	1	3†
5	4	1	1
7	5	2	8† 4

* Same comb used
† Slept w th infected male

observed It is of interest however that of 109 females infected 37 or 34 per cent claimed to have had only one or a few ringworms on the body that disappeared within a few weeks following home med cations This high incidence of tinea corporis is of course entirely out of line with that generally accepted Tinea corporis was of further interest to us in that in only one family was a skin infection incriminated as a source of additional infections This we anticipated because of the short duration of the disease and the un doubtedly fewer organisms being shed We realized therefore we could not use tinea corporis as a focus for reinfection in our immunological stu l es

Only single infections occurred in 130 of the familie providing furtl er evidence of the low infectivity of *M and u ni* Forty of the e famil es had seemingly ideal situations for cros infection in that they had su cej tible males as well as infected males with noninflammatory tinea capitis of long duration to serve as sources of infection However questioning revealed that 30 of these 40 families exercised at least some precaution to prevent sprea l of the

disease to other members of the family Five enforced rather stringent procedures that included daily shampooing and clipping of the scalp as well as isolation of the sleeping quarters The infected boys of 18 of the families slept alone and 7 families had the infected person sleep at the foot of the bed or wear protective clothing or both However, 10 of the 40 single-infection families with susceptible males did nothing to prevent cross infections In TABLE 1 may be seen the age and numbers of the susceptible males of the 10 families and duration of the focus of infection as estimated by period of fluorescence sleeping and other habits of the siblings Cross infection did not

TABLE 2

A DESCRIPTION OF FOUR FAMILIES WITH ONE OR MORE MALES WITH NONINFLAMMATORY TINEA CAPITIS CAUSED BY *MICROSPORUM AUDOUINI* AND ONE MALE WHO ESCAPED INFECTION

4 Susceptible Males Escaped Infection

Jordon family 4 boys
3 infected (ages 4 7 10) >1 year
1 noninfected (age 6) all 4 slept together
Rhodes family 4 boys
3 infected (ages 3 4 6) > 1 year
1 noninfected (age 9) slept with 2 infected boys
Dixon family 4 boys
1 infected (age 6) >1 year
3 noninfected 1 (age 7) slept with infected boy
Napoleon family 4 boys
3 infected (ages 6 3 7) >6 months
1 noninfected (age 7) slept with 3 year-old infected boy

TABLE 3

DESCRIPTION OF THREE LARGE FAMILIES WITH SUSCEPTIBLE MEMBERS NEARLY ALL OF WHOM ACQUIRED TINEA CAPITIS CAUSED BY *MICROSPORUM AUDOUINI*

Green family 8 children (4 boys 4 girls)
7 infected (1 girl age 6 not infected)
Hurst family 7 children (2 boys 5 girls)
5 infected (2 girls ages 3 and 10 not infected)
Harvey family 5 children (3 boys 2 girls)
All infected

occur even though in 2 instances a single comb was used by all members of the family and prolonged contact, such as sleeping together, occurred among all of the families In one family, 4 children 1 of whom was infected slept 4 abreast in one bed It would seem that most of the members of these 10 families were naturally resistant' or that contact such as sleeping together did not provide a suitable means of transferring an infective inoculum However a study of 49 families with a total of 150 boys indicated that sleeping together might serve as an effective means of providing conditions conducive for cross infection Of the 150 boys in this group, 113 had noninflammatory tinea capitis Forty nine obviously acquired their infections from outside the family but 64 cases were probably acquired as a result of cross infection Of these 64 boys 45 or 70 per cent slept regularly with an infected member of

the family and thus may have acquired their infections in this manner Nineteen or 30 per cent did not sleep with an infected person and must have become infected through other contact Of perhaps greater interest of the 37 susceptible males that escaped infection 33 or 89 per cent slept alone or with a noninfected individual and only 4 slept with an infected person Thus it appears that the chance of escaping infection is greatest if sleeping with an infected person is avoided TABLE 2 shows the conditions of exposure of the 4 boys who escaped infection even though they regularly slept with an infected person These observations attest to the usual low infectivity of *M audouini*

It is worthy of note that in only 3 of 257 families with at least 1 member infected with *M audouini*, did all or nearly all susceptible children including females have tinea capitis A description of these families is shown in TABLE 3 The high rate of infectivity observed with this small percentage of the families studied was indeed exceptional in that never did as many members of

TABLE 4
RESISTANCE TO REINFECTION OF RECOVERED NONINFLAMMATORY TINEA CAPITIS PATIENTS

Noninfected		Re-exposure to noninflammatory tinea capitis	
Sex	Age	Period	Sex and age of infected child
M*	5	12 months	F* 3 M* 2
M	10	15 months	3
F	10	6 months	M 12
M	11	12 months	M 5
M	4	2 months	M* 9
M*	3		
M*	6		
M*	9	16 months	M* 9 M 3

* Sleep together

a family, including even the females, become infected We believe these were most likely strains of unusual infectivity

In only 8 of the 257 families studied was there certain re-exposure of individuals recovered from tinea capitis pointing up the difficulty usually encountered when attempting to determine an immunological response under natural conditions These patients either had been cured by one of the many nostrums prescribed in our clinic or had cleared spontaneously TABLE 4 shows the conditions of re exposure None of them became infected a second time

With the advent of griseofulvin an opportunity was afforded to search more thoroughly for evidence of immunity following infection The first indication was the observation that some of our patients who were clinically cured and nonfluorescent retained viable organisms for some few months thereafter Until very recently we had felt this represented loose spores surviving in the hair of the previously infected individuals It was not inconceivable that fungus spores notorious for their survival properties might well remain for some months following only oral medication, especially since these patients

often practiced very poor hygiene and since the numbers of colonies observed in culture were so often very few in number. A description of these patients and the numbers of colonies found on each of the cultures taken at regular intervals following loss of fluorescence is shown in TABLE 5. We had believed failure to become reinfected after exposure to these residual fungi indicative of

TABLE 5
RESIDUAL FUNGI IN ORALLY TREATED PATIENTS WITH TINEA CAPITIS (*M. AUDOUINI*)

Patient No.	Griseofulvin dosage	Nonfluorescent since 1959	No. colonies*
1	1 gm Q3Dx28†	July	TNC 0 10 0 1
2	3 gm 1 dose	October	TNC 6 1 0 0
3	1 gm Q3Dx28	July	TNC 313 0 5 34 0
4	3 gm 1 dose	September	TNC 27 TNC 6
5	3 gm 1 dose	August	TNC 20 0 TNC 26 0

* Cultures collected at various times since loss of fluorescence.
† Q3Dx28 every 3 days for 28 days.
TNC: Number of colonies too numerous to count.

TABLE 6
RESISTANCE TO REINFECTION OF RECOVERED PATIENTS WITH NONINFLAMMATORY TINEA CAPITIS TREATED WITH GRISEOFULVIN

Family no.	Received griseofulvin		Re-exposure to fluorescent noninflammatory tinea capitis
	Sex	Age	
1	M*	8	8 months to M* 1 age 7
	*M**	*5*	*8 months to F* 2 age 3*
2	M*	6	6 months to F* age 3
3	M*	11	6 months to M age 10
4	M*	6	6 months to M* age 9 and M* age 4
5	M	5	6 months to F age 2 M age 3 and M* age 9
	M	5	
	M*	6	
	M*	7	
6	M*	9	6 months to M* age 7
7	M	9	M age 6
8	M*	7	M* age 5
9	M		6 months to M age ?
10	M*	7	M* age 9
Total 15			

* Slept together. F: female; M: male.

a degree of immunity following recovery from infection. However some doubt is cast on this reasoning by the recent observation of Rosenthal and his associates[7] that the skin of guinea pigs may remain infected with *T. mentagrophytes* after griseofulvin treatment even after the hairs are rendered free of fungi. We are presently trying to determine whether these cultural isolations in clinically recovered patients represent surviving but not infecting spores or latent infections. If the latter is true, the failure to become clinically

diseased again could not be considered as evidence of immunity However none of fifteen culturally as well as clinically cured patients thus far exposed to foci of infection have become infected a second time In two instances we have isolated *M audouini* in small number from their scalps but this is not surprising inasmuch as similar isolations have been made from their home environs and the observation merely supports the thesis that these patients were re exposed The conditions of these re exposures described in TABLE 6 seemed as intense as could be devised short of deliberate reinoculation Most of the foci for reinfection were males in age groups similar to those being exposed, thus presumably providing ample opportunity for vigorous contact Also, most of those being exposed slept regularly with an infected individual By coincidence family number 5 was the Green family shown in TABLE 3 as one of the few families infected with what is probably a strain of unusually high infective properties Admittedly, a far larger number of individuals escaped first infection in our study of 257 families casting some doubt on the failure of these 15 to become reinfected as being evidence of immunity However those escaping infection among the 257 families represented only a small percentage of the whole whereas the escape of these 15 even though a small group, was uniform It is our opinion these individuals became resistant as a result of a previous infection Whether this represents an immunity due to an as yet undetected antibody is yet to be resolved However it is not inconceivable that an infectious agent could induce a specific and protective cellular alteration Finally, it is significant that griseofulvin therapy apparently did not block this mechanism

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CAPSULAR REACTIONS OF *CRYPTOCOCCUS NEOFORMANS**

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The work of several groups has established the fact that the capsules of *Cryptococcus neoformans*[1-5] and related species[6-8] are predominantly polysaccharide, however, much remains to be learned about the finer details of capsular structure, the relation of the capsule to other cellular components, its mechanism of synthesis, and its functions The resolution of these problems undoubtedly will necessitate the use of considerable imagination plus a variety of chemical, immunological, and cytological methods

One approach that has been particularly fruitful in studying the capsule in its normal hydrated state is the use of specific antibodies directed toward capsular components An excellent example of the possibilities inherent in this approach is seen in the work of Tomcsik and his associates,[9,10] who have demonstrated a complex structure in the capsule of certain strains of *Bacillus megaterium* through the use of antibodies specific for a glutamyl polypeptide and for a polysaccharide

The existence of antibodies against the capsule of *C neoformans* was first demonstrated by Neill *et al*[1] and, shortly thereafter, our work[11] disclosed that this species was antigenically heterogeneous due to differences in capsular polysaccharides of various strains Although the reaction between antibody and capsule is similar to the "Quellung" reaction of pneumococcus, it is generally referred to as the "capsular reaction"[10] (CR) since there is no swelling of the capsule when it reacts with antibody[1,5,6] Our results agree with those of Tomcsik,[10] who describes the reaction as " precipitation which renders the capsule visible, usually without changing its size and shape "

More recently we have described another capsular reaction in which the capsular outline of *C neoformans* is revealed by a basic polysaccharide rather than specific antibody This basic polysaccharide, which has been designated APS, is a soluble polygalactosamine isolated from *Aspergillus parasiticus* by Blumenthal and his associates[13] and by Distler and Roseman[14] It contains both acetylated and free amino groups, and it is presumably due to the latter that it reacts with the acidic capsular polysaccharide of *C neoformans*[15] Although the capsular reaction due to APS is less specific than that due to antibody it is nevertheless important for the information it provides on the ability of the cryptococcal capsule to function as a polyvalent anion The APS reaction appears to be similar in principle to the nonspecific reactions of proteins with microbial capsules (Tomcsik[9,10]), however, the APS molecule possesses fundamental structural differences from the proteins used by Tomcsik since it is a basic polysaccharide The effective *p*H range of the APS reaction is also much wider than one finds with proteins, although Tomcsik found that lysozyme reacted weakly with cryptococcus between *p*H 2 and 5

The present report summarizes our work on the APS reaction as well as some

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further observations on the reaction between the cryptococcal capsule ar specific antibody

Methods

C neoformans, strain 6 (Type A) and strain 15 (Type B) were used for thes studies Cultures were grown on neopeptone glucose broth enriched witl thiamine[3,5] or on the same medium solidified with 2 per cent (w/v) agar I o some experiments cells were harvested from peritoneal exudates of experi mentally infected white mice Preparation of polysaccharides,[3,4,15] anti serum[11,14] and APS solutions[15] has been described elsewhere Quantitative precipitin determinations by micro Kjeldahl analysis of antibody nitrogen were performed by methods described in Kabat and Mayer[7] and upernates from these precipitations were tested for residual antibody by determining the capsular reaction (CR) titer on each tube CR titers with antibody or with APS were determined as outlined in earlier reports[1,5] Cryptococcal cell suspensions used in the CR were centrifuged and rinsed 5 times before each experiment and standardized at 10^7 or 2×10^7 cells ml

For certain experiments, cryptococcal cells were fragmented by mixing a thick slurry of cells in saline with an equal volume of 0 2 mm glass beads and subjecting this mixture to high speed stirring in a Virtis 45 homogenizer The sample-container was immersed in a melting ice bath and samples were re moved after 5, 10-, 20-, and 30-min treatment at rheostat setting no 60

Results

If the CR with APS was conducted in saline solution or in M 20 phosphate buffer at a *p*H of 6 or 7, it resembled the antibody CR quite closely except that it was slightly less intense (FIGURE 1*a* and *b*) If the APS reaction was conducted in distilled water or at extremes of *p*H there wa considerable shrinkage and distortion of the capsule (FIGURE 1*c* and *d*) As might be ex pected cells that have undergone a CR with APS also agglutinated The CR is usually read after 30-min incubation at room temperature while agglu tination is read after the tubes have been stored for an additional 18 hours at 0 to 4°C[14] In a strong agglutination the cells were firmly adherent to the test tube, but could be removed by scraping with a stirring rod TABLE 1 shows that titers obtained in the agglutination test were not significantly higher than the CR The 3 serologic types of *C neoformans* could not be differentiated by the APS reaction

Additional evidence that the APS reaction lacks the specificity of the anti body reaction was found in a study of precipitation reactions If the soluble S polysaccharide of *C neoformans* was mixed with APS in 0 15 M NaCl pre cipitation resulted[15] This reaction was obtained not only with the three types of *C* polysaccharide, but also with the capsular polysaccharides of pneu mococcus Types 2 and 3, as well as with gum acacia Since these polysac charides all contain carboxyl groups it seemed likely that precipitation was the result of combination between such groups and the free-amino groups of APS Dextran which does not contain carboxyl groups failed to react while proteins such as rabbit serum and rabbit gamma globulin would precipitate

with APS or would block the CR between APS and cryptococcal cells (E. E. Evans, unpublished data).

The reaction between APS and the capsule of *C. neoformans* can occur within a wide *p*H range; however, the range may be more restricted with a single buffer system as shown in TABLE 2. For example, with citrate buffer the reaction is strong at *p*H 3 and 4, weaker at *p*H 5, and negative at *p*H 6, whereas

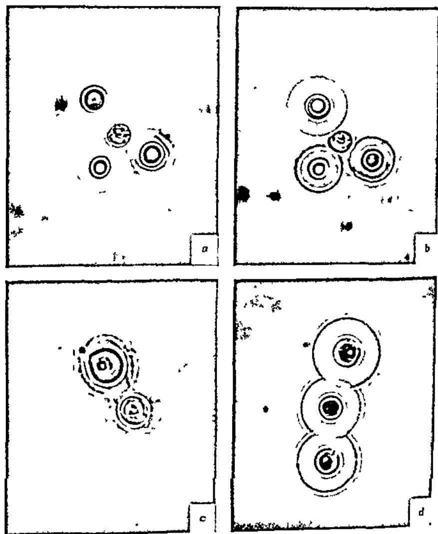


FIGURE 1. (*a*) The capsular reaction (CR) between APS and cryptococcal cells in 0.15 *M* sodium chloride solution. (*b*) The same cells shown in FIGURE 1*a* after antibody was applied to produce a specific CR. (*c*) The CR between APS and cryptococcal cells in distilled water. Note the shrinkage of the capsule *in contrast to* FIGURE 1*a*. (*d*) A group of cells from the same preparation shown in FIGURE 1*c* after the addition of antibody to produce a specific CR.

the reaction in phosphate buffer is strong at *p*H 5 and 6 weaker at *p*H 7 and negative at *p*H 8 This suggests that concentration of buffer ions other than the hydrogen ion is important

Under certain conditions, the APS bond is quite stable and cryptococcal cells retain a visible APS CR even after repeated centrifuging and rinsing TABLE 3 shows the effect of 5 rinses with APS free buffers on cells that have been treated with 50 µg/ml of APS for 30 min at 3 different *p*H levels With citrate buffer, *p*H 4, and phthalate buffer, *p*H 6 and APS bond survived at

TABLE 1
AGGLUTINATION AND CR TITERS OF *C NEOFORMANS* PLUS APS IN 0 15 *M* SODIUM CHLORIDE

Reaction	APS µg/ml					
	25	12 5	6	3	1 5	0 8
CR	CR	(CR)	(CR)	−)	−	−
Agglutination	+	+	+	±	±	−

Strength and variability of the capsular reaction are indicated in decreasing order by the symbols CR (CR) (−)

TABLE 2
CR OF *C NEOFORMANS* WITH APS IN VARIOUS BUFFER SYSTEMS

*p*H	Buffer (*M*/20)			
	Citrate	Phosphate	Veronal	Glycine NaOH
3	CR			
4	CR			
5	(CR)	CR		
6	−	CR		
7		(CR)	CR	
8		−	CR	
9				CR
10				(CR)

See footnote to TABLE 1

least 5 rinses, but with HCl KCl buffer at *p*H 2 the CR disappeared during the first rinse Incidentally, the reaction mixture designated Rinse 0 in the table did not reach the buffer *p*H of 2, but was approximately 2 3 to 2 4 and when the basic APS was removed during the first rinse with *p*H 2 buffer, the *p*H would be expected to decrease to approximately 2.0 at which point carboxyl groups are probably not dissociated One may therefore view the extinction of the CR during the first rinse as a replacement of APS with hydrogen ion That APS was actually replaced could be verified by taking the cells from the first *p*H 2 rinse and exposing them to citrate buffer at *p*H 4 a system in which a strong CR is obtained When this was done no CR resulted, indicating that APS had been replaced Such a result could have been due to loss of the capsule, but India ink disclosed that the capsule was still present, and a fresh

preparation of APS in pH 4 citrate buffer showed that it was still capable of reacting

Thus it was possible to reverse the APS reaction by replacing it with other cations Of the 2 cations present in the pH 2 buffer, H^+ was much more effective in producing reversal since HCl could do so in a concentration of 0 025 M, while approximately 4 M KCl was required to cause extinction of the CR It should be noted however that if KCl was added before APS was mixed with cells the reaction was inhibited by concentrations as low as 0 5 M

TABLE 3
EFFECT OF REPEATED RINSING ON THE CR PRODUCED BY APS

Rinse No.	Buffer used for rinse		
	Phthalate pH 6	Citrate pH 4	HCl KCl pH 2
0*	CR*	CR*	CR*
1	(CR)	CR	—
2	(CR)	CR	—
3	(CR)	CR	—
4	(CR)	CR	
5	(CR)	(CR)	

* APS was added (50 μg /ml) only to Rinse 0

TABLE 4
INHIBITION OF THE CR BETWEEN *C NEOFORMANS* (TYPE A) AND APS AFTER THE ADDITION OF DISSOLVED SA TO CELLS IN VARIOUS BUFFER SYSTEMS

Buffer (M/20)	SA added (μg /ml.)			
	0	10	100	1000
HCl KCl (pH 2)*	CR	CR	(CR)	—
Citrate (pH 4)	CR	CR	—	—
Phosphate (pH 6)	CR	CR	—	—
Veronal (pH 8)	CR	CR	—	—

* Final pH of reacting mixture was 2 3 to 2 4 cell concentration 5 × 10⁶/ml APS concentration 50 μg /ml

The APS reaction then may be visualized as a reaction between a polyvalent anion (the S polysaccharide of *C neoformans*) and a polyvalent cation (the APS polysaccharide) It is important because it emphasizes the role of the acidic capsular polysaccharide in binding various ionized substances and it suggests that the capsule may play a role in cell permeability due to its anionic nature

We do not yet have a complete picture of the chemical structure of the cryptococcal capsule however several laboratories have isolated the acidic S polysaccharide that reacts with APS Drouhet and his associates[2] were the first to describe the hydrolytic products as xylose mannose and glucuronic acid and our results with the S polysaccharide have confirmed this although a second polysaccharide is present in cultural supernates [5]

Certain inhibition and absorption experiments provide some support for the hypothesis that the S polysaccharide is the major ingredient of the *Cryptococcus* capsule If, in the CR with APS the S polysaccharide was mixed with a cell suspension before APS was added the dissolved S competed with the capsule for APS, and the extent of the inhibition by Type A polysaccharide (SA) can be seen in TABLE 4 Inhibition by an excess of SA was slightly less

TABLE 5
CR ON SUPERNATES OF QUANTITATIVE PRECIPITIN DETERMINATION (TYPE B

SB added (mg)	Antibody N precipitated (mg)	Supernate test	
		PPN	CR titer
0 00	0 00	Excess Ab	50
0 05	0 19	Excess Ab	40
0 10	0 30	Excess Ab	20
0 30	0 46	No Ab or S	<8
0 60	0 54	Excess S	<8
1 00	0 59	Excess S	<8
1 25	0 60	Excess S	*

* Not tested

TABLE 6
INHIBITION OF CR BY MIXING S WITH CELL SUSPENSION BEFORE THE ADDITION OF ANTIBODY

Antiserum	Cell type	S added		CR titer
		Type	mg./ml	
Type A	A	SA	0	40
			0 31	10
			0 63	0*
			1 25	0
Type B	B	SB	0	20
			0 31	10
			0 63	0
			1 25	0

* A titer of 0 indicates failure to react at 1 4 the lowest serum dilution possible final cell concentration 2 5 × 10^4/ml

effective with pH 2 buffer (final pH 2 3 to 2 4), otherwise complete inhibition was obtained with 100 μg of SA per 5 × 10^6 cells per 50 μg of APS The fact that such inhibition can occur emphasizes the importance of rinsing encapsulated cells immediately prior to their use in an experiment Since the APS reaction is less specific than that due to antibody, more significance should be attached to the results presented in TABLES 5 and 6

If the S polysaccharide is allowed to precipitate with antibody and the supernates from each tube are tested for reactivity in the capsular reaction, it is found that the capsular reaction titer diminishes as more antibody is removed until it becomes negative near the equivalence zone (TABLE 5) As

in the APS reaction inhibition of the specific capsular reaction can be observed by adding dissolved S to suspensions of cells before antibody is added showing that S competes with the capsule for the antibody (TABLE 6)

These data suggest that the S polysaccharide is the major component of the region near the capsular surface Since antibody and APS appear to react only at the surface of the capsule we cannot rule out the possibility of other capsular components in a zone adjacent to the cell wall Also the inhibition and absorption experiments would fail to reveal impurities that do not react with the antibody or APS It should be recalled that extracellular starch[6] can be synthesized by *C neoformans* but that it appears only in growth media in which the *p*H drops below 5 Under the usual conditions of cultivation starch does not appear to be synthesized and thus it need not be considered as contributing to the structure of the capsule

Cryptococcal cells growing in fluid cultures elaborate soluble capsular polysaccharide into the supernate while the culture is growing When the cells are killed and rinsed they can be stored for prolonged periods in water or saline without loss of capsule and can be subjected to repeated alternate rinsing and centrifugation without apparent reduction in size of the capsule In the absence of cytological evidence to the contrary it might seem that the outer layer of the capsule was covered by a membrane That this is not the case may be seen in experiments in which cells were fragmented by bombardment with 0 2 mm glass beads in a high speed homogenizer After 10 to 30 min of such treatment cells in various stages of fragmentation could be observed under the microscope In FIGURE 2 is seen a typical group of cells after 10-min treatment Antibody has been added to disclose the boundary of the capsule It is apparent that even when the capsule and cell wall have been sliced through the capsule does not flow This is evidence against a capsular membrane and it suggests that the capsule is a thick gel one that might even be called a tough gel since it can withstand considerable mechanical trauma

At present it is not known whether the firmness of the capsule is due merely to concentration of the S polysaccharide or perhaps to some complex between the S polysaccharide and one or more other substances however evidence has been obtained that the S polysaccharide alone can produce a firm gel when mixed with water Artificial capsules have been prepared by dissolving the S polysaccharide in water or saline at concentrations of 10 20 40 and 80 mg/ml In the case of the Type A preparation used (SA) the contents of each test tube above the 10 mg level set up as a firm gel These gels could be rinsed 100 times with saline solution without dissolving although there was some degree of swelling When washed gels of this type were overlaid with antibody a precipitate resulted but it was confined to a fine line at the interface After the preparations were allowed to stand at room temperature additional precipitation occurred in the supernatant fluid but in no case did it extend into the gel Similar results were obtained with APS These experiments help to explain why the specific capsular reaction appears to produce only an interfacial line near the capsular surface for if the concentration of S polysaccharide in the intact capsule approached the concentration in our

artificial capsules, precipitation within the capsular sub t in ... out due to inhibition by the great excess of antigen in r ... of antibody that might diffuse into the gel This is in agr ... scopic observations made on the CR of encapsulated crypt ... unpublished data)

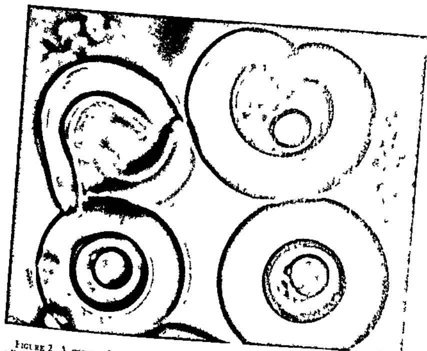


FIGURE 2 A group of cryptococcal cells showing vary ng legrees ... cell wall an l capsule following high speed stirring with 0 2 n m g ass be ... been ad led to show the location of the capsular gel

Summary and Conclusions

Some recent observations were presented on the cap ul r rea tions (CR) of *C neoformans* with specific antibody and with the polygalactosamine (APS) from *Aspergillus parasiticus* Both varieties of the CR app ared to have some potential value as cytological tools Under certain conditions the CR pro duced by APS resembled the CR produced by antibody but when the APS reaction occurred in distilled water or at extremes of *pH* considerable shrinkage and distortion of the capsule were observed The APS reaction appeared to be a combination of a polyvalent cation (APS) with a polyvalent anion (*Cryp tococcus* S polysaccharide) In contrast to the reaction between antibody and the capsule, the APS reaction appeared to be relatively non pecific since it occurred with all compounds tested that are known to possess carboxyl groups Studies with APS suggested that the capsule of *Cryp ococcus* as

well as other microbial capsules, might have a role in the binding of various substances that possess cationic groups Because of its anionic nature and potential role in ion exchange processes, the capsule could exert some influence on permeability

Inhibition and absorption data were compatible with the hypothesis that the S polysaccharide is the major ingredient of the *Cryptococcus* capsule At present however, one cannot exclude the possibility of other capsular ingredients in a zone adjacent to the cell wall or of substances that do not react with APS or antibody Even if subsequent work should verify the fact that the S polysaccharide is the only "resident" ingredient of the capsule it would seem not unlikely that various substances that possess cationic groups could be located transiently" within the negatively charged capsule

Experiments in which the cell was fragmented mechanically disclosed that the capsule was quite resistant to disintegration and that it appeared to be a firm gel Gels with similar properties (artificial capsules ') could be made in the test tube by dissolving relatively high concentrations of type A polysaccharide (SA) in aqueous media Such artificial capsules were also used to study the reaction of the capsular gel with antibody As a result of such observations together with microscopic studies of the natural capsule (E E Evans, unpublished data), it is suggested that the CR produced by antibody is a thin line of precipitate formed at the interfacial gradient between the capsular gel and the surrounding fluid

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ANTIGENS OF *BLASTOMYCES*

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The material to be reported stems from interest in the hypothes[illegible] agents more specific than those available might be prepared for use in ser[illegible] and skin test reactions involving systemic mycotic pathogens Th[illegible] m[illegible] of specificity of skin test reagents is of prime importance becau[illegible] demonstrating that cross reactions may occur among individu[illegible] to histoplasmin, blastomycin, coccidioidin, and Emmonsin [1-5]*

Martin has presented the results of wide ranging studies on variou[illegible] *myces dermatitidis* antigens [6-8] Soluble antigen obtained by sonic [illegible] of yeast phase cells of *B dermatitidis*, yielded as satisfactory a com[illegible] fixation test antigen as entire yeast phase cells but had the adv[illegible] solubility and ease of storage A polysaccharide precipitated from ye[illegible] cell extract was found to be serologically active as well as capable of [illegible] a limited degree of resistance to disease in mice The crude extract[illegible] were not found to be of significant value as skin test antigens We have furth[illegible] explored these areas and, in this report, present aspects of strain variation in *B dermatitidis* and the comparison of serologic skin reactive and immunogen[illegible] properties of polysaccharides prepared from different strains of this organism

Constancy of Virulence and Strain Variation in B dermatitidis

Benham has stated [9] "Pathogenicity [of *B dermatitidis*] seems to be fixed in character, for, after years on artificial media this fungus will reproduce the disease in animals"

TABLE 1 gives typical results of mouse (albino *Mus musculus*) LD_{50} determinations with 3 strains of *B dermatitidis*

Strain 410 originally isolated from surgically excised pulmonary tissue at Tulane University, New Orleans, La and sent to us in 1952 by M L Littman, has been employed extensively by our group As noted in TABLE 1 the organism has maintained an intravenous LD_{50} for 21 days, of about 200 population units

The term "population unit" is defined as individual cells, budding cells, or clumps of cells, each counted as a unit The organisms have been maintained on 10 to 15 per cent human blood agar slants, containing penicillin and strepto-

* Evidence referred to in this publication has made necessary a change of the name of the organism formerly known as *Haplosporangium parvum* to *Emmonsia parva* A second species described by Emmons and Jellison in this new genus is called *E crescens* The new nomenclature requires revision of the term employed for the filtrate antigen that should now be called Emmonsin rather than haplosporangin

mycin, at 37° C. The strains were usually transferred at weekly intervals although occasionally as much as 4 weeks passed between transfers.

Inocula were prepared by harvesting the yeast phase growth from a 96 hour blood agar slant into broth. The broth suspension is filtered through sterile glass wool and a quantitatively diluted aliquot of the suspension is counted in a hemocytometer. This count is employed to make the estimate of the broth suspended population and of the dilutions designed to contain the numbers of population units to be injected. All injections have been made intravenously (I.V.) into a tail vein of albino *Mus musculus* obtained from local sources.

TABLE 1

MOUSE LD_{50} DETERMINATIONS WITH *B. DERMATITIDIS*

Strain and date of test	No. of organisms injected	Mortality ratio	LD_{50} (population units)
410 10/15/51	100	5/10	155 (77 to 310)*
	500	7/10	
	1000	9/10	
	2000	10/10	
2/19/52	10	0/8	285 (119 to 684)
	100	3/8	
	1000	5/8	
9/10/57	17	5/24	115 (32 to 406)
	170	9/18	
	1700	14/16	
380	10^4	0/10	—
	10^5	0/10	—
	10^6	0/10	—
6052	10^4	0/10	8.5×10^5 (0.33 to 2.2×10^6)
	10^5	1/10	
	10^6	4/10	

* Ninety five per cent confidence limits.

The I.V. challenge with *B. dermatitidis* produces predominantly lung lesions from which the organisms are readily cultured.

The LD_{50} value of about 200 population units for strain 410 was maintained over the 6-year period shown. This observation is similar to Benham's.[9]

Variation in virulence of strains is considered in the remainder of TABLE 1. Strain 380, also from the Tulane collection, was isolated from a fatal case of blastomycosis. This organism has been uniformly nonlethal in doses of 1×10^6 population units. Strain 6052, received from Emmons' collection, has maintained a 21-day LD_{50} of approximately 10^6 population units since 1955.

The statistical analyses have been carried out employing the methods of Wilcoxon and Litchfield.[10]

The discovery of variation in animal virulence of strains of *B. dermatitidis* is not unexpected, that is, virulence differences among strains of the same fungus organism have been reported by others. For example, Howell and Kipple[11]

and Drouhet and Schwarz[12] have reported virulence differen[illegible] *sulatum* Friedman *et al*[13] have demonstrated virulen[illegible] strains of *C immitis*, and Salvin *et al*[14] have shown differ[illegible] for mice among strains of *C albicans*

It may be concluded that the three strains of *B dermatitidis* [illegible] originally from human infections and maintained in yea[illegible] culture media for prolonged periods of time showed [illegible] although stable virulence characteristics for mice by the meth[illegible] described The three strains were found to be indistingu[illegible] cally in either yeast or mycelial phase grossly and micro[illegible] that conflicting reports concerning effects observed in anim[illegible] *B dermatitidis* could be due to differences in the virulen[illegible] organism used rather than to the effect being measured

Serologic Characteristics

Yeast phase cell free extracts were prepared from org[illegible] biotic containing blood agar plates and in broth Both ty[illegible] incubated for 3 to 7 days at 37° C The agar grown or[illegible] to the broth suspensions, and all were killed by addition [illegible] per cent final concentration, 24 hours at 37° C [illegible] tion as determined by test of viability on agar Each [illegible] and then transferred to a heavy glass centrifuge bottle [illegible] The bottle was placed on ball mill rollers and run at sl[illegible] at 5° C Cellular debris was centrifuged off at high speed [illegible] supernate represented cell free extract (CFE)

Polysaccharides were extracted by the methods of Heidell[illegible] modified by local circumstances[15] This procedure gave goo[illegible] saccharides from both yeast cell filtrate broths and CFE [illegible] fraction was prepared by saturating CFE with ammonium sulf[illegible]

Larger yields of brownish to grey colored polysacchar[illegible] from yeast phase broth filtrates than from CFE A statem[illegible] 1953 report[7] is appropriate " antigenically active polysaccharid[illegible] the surfaces of yeast phase cells of *B dermatitidis* which diffu[illegible] into the surrounding fluid " For example per liter of original material w[illegible] obtained 5 74 gm from broth versus 510 mg from CFE of *B dermatitidis* strain 380 The control, that is, uninoculated but incubated broth yielded 80 mg of material Seven day broth cultures of strain 410 yielded 1 4[illegible] gm [illegible] and strain 6052 yielded 3 92 gm /l Yields of similar nature and appearance have been obtained with 3 strains of *H capsulatum* and 1 strain each of *Sporotrichum schenkii* and *Coccidioides immitis* The control material obtained was serologically inactive and yielded no skin test reactions in animals or in [illegible] human volunteers injected with 100-μg amounts

Attention has been given to some chemical characteristics of these polysaccharide substances

The hydrolyzed *H capsulatum* and *B dermatitidis* materials each contained 1 ascending migrating constituent in 1-dimensional paper chromatograms These carbohydrates, when developed into ultraviolet light [illegible]

mycin at 37° C The strains were usually transferred at weekly intervals although occasionally as much as 4 weeks passed between transfers

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in the same relative position as the control hexose (dextrose) spots No pentose or disaccharide was detected Protracted hydrolysis yielded 75 to 90 per cent reducing substances, hexoses measured 50 to 59 per cent Iodine and biuret tests of solutions of the materials were negative, Molisch tests were positive Small amounts of nitrogen were present in each preparation A guess concerning structure of these polysaccharides is that they are largely or entirely hexose polymers The nitrogen content may represent contamination or a structure containing an occasional hexosamine moiety

Examination of the extracted polysaccharides by the Ouchterlony[16] method of agar plate double diffusion has been carried out Central wells contained rabbit antiserum of known specific reactivity, peripheral wells contained polysaccharides about 100 µg/ml, from *H capsulatum*, *B dermatitidis*, *C immitis* and *Sp schenkii*, also wells containing dextran and saline were filled as controls The only reactions noted were with the homologous system in each case

No opportunity has been available to carry out tests with this technique and sera from infected humans Low titer complement fixing antibody from infected guinea pigs has been uniformly negative by the Ouchterlony procedure

J H Schubert (personal communication) examined these polysaccharides at the Communicable Disease Center Chamblee, Ga and found that the *H capsulatum* material is not comparable with antigen employed there with serum from proved cases of persons with histoplasmosis *B dermatitidis* antigen was comparable in concentrations of 100 µg/ml and *C immitis* in concentrations of 10 000 µg/ml Positive human serum against *Sp schenkii* was not available but employing rabbit antibody, Schubert found that 50 µg/ml of polysaccharide reacted similarly to antigen employed by the CDC

We have had the opportunity to examine the serum from an individual (M W) with active histoplasmosis who died in the hospital of complications of noninfectious disease The serum yielded a titer of 1 16 to 1 32 with both CFE and *H capsulatum* polysaccharide, 50 µg/ml, but no reactions with the other available antigens

When mycelial filtrate antigens that is, histoplasmin blastomycin, and coccidioidin were examined by agar plate double diffusion against the rabbit CFE antisera only one band of homologous precipitate was noted Since the filtrates contain protein as well as polysaccharide, reasons for observing but one band are open to speculation and further investigation The suggestion has been offered that analysis by Oudin procedure[15] would yield more sensitive results

Results obtained by complement fixation with rabbit antibody from animals immunized with CFE are shown in TABLES 2 and 3 The results of TABLE 2 were obtained with polysaccharide antigen, the result of TABLE 3 with CFE antigen In each case the micro Kolmer complement fixation method was employed It is apparent that cross reaction characterized these results For example anti *H capsulatum* antiserum cross reacted to titer with *B dermatitidis* polysaccharide However, a degree of discrimination is apparent in these results a 1 40 dilution of anti *H capsulatum* antibody showed no reaction

in the same relative position as the control hexose (dextrose) spots. No pentose or disaccharide was detected. Protracted hydrolysis yielded 75 to 90 per cent reducing substances, hexoses measured 50 to 59 per cent. Iodine and biuret tests of solutions of the materials were negative, Molisch tests were positive. Small amounts of nitrogen were present in each preparation. A guess concerning structure of these polysaccharides is that they are largely or entirely hexose polymers. The nitrogen content may represent contamination or a structure containing an occasional hexosamine moiety.

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No opportunity has been available to carry out tests with this technique and sera from infected humans. Low titer complement fixing antibody from infected guinea pigs has been uniformly negative by the Ouchterlony procedure.

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We have had the opportunity to examine the serum from an individual (M. W.) with active histoplasmosis who died in the hospital of complications of noninfectious disease. The serum yielded a titer of 1:16 to 1:32 with both CFE and *H. capsulatum* polysaccharide, 50 μg/ml, but no reactions with the other available antigens.

When mycelial filtrate antigens, that is, histoplasmin, blastomycin and coccidioidin, were examined by agar plate double diffusion against the rabbit CFE antisera, only one band of homologous precipitate was noted. Since the trates contain protein as well as polysaccharide, reasons for observing but band are open to speculation and further investigation. The suggestion n offered that analysis by Oudin procedure[16] would yield more sensitive

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TABLE 2
COMPLEMENT FIXATION TITERS RABBIT ANTIBODY VERSUS POLYSACCHARIDE

Rabbit antibody dilutions	1	Polysaccharide antigen µg/ml																			
		H capsulatum					*B dermatitidis*					*C immitis*					*Sp schenkii*				
		100	50	25	12	0*	100	50	25	12	0	100	50	25	12	0	100	50	25	12	0
anti *H capsulatum*	10	4	4	4	4	2	4	4	4	4	2	4	4	4	4	2	4	4	4	4	2
	20	4	4	4	3	0	4	3	3	0	0	4	3	1	1	0	4	4	4	4	0
	40	2	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
anti *B dermatitidis*	10	4	4	4	4	2	4	4	4	4	2	4	4	4	3	2	4	4	4	3	2
	20	3	2	2	2	0	4	4	3	2	0	3	2	1	1	0	4	4	2	1	0
	40	1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
anti *C immitis*	10	4	4	4	4	3	4	4	4	4	2	4	4	4	4	3	4	4	4	4	3
	20	4	4	4	4	2	4	4	3	3	2	4	4	4	4	2	4	4	4	3	2
	40	1	1	1	1	0	3	0	0	0	0	4	4	3	2	0	2	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
anti *Sp schenkii*	10	4	4	3	3	0	4	4	3	3	0	3	3	2	2	0	4	4	3	3	0
	20	2	0	0	0	0	2	1	0	0	0	1	0	0	0	0	3	3	0	0	0
	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Saline control, 0 no fixation, 4, complete fixation

in the same relative position as the control hexose (dextrose) spots No pentose or disaccharide was detected Protracted hydrolysis yielded 75 to 90 per cent reducing substances, hexoses measured 50 to 59 per cent Iodine and biuret tests of solutions of the materials were negative, Molisch tests were positive Small amounts of nitrogen were present in each preparation A guess concerning structure of these polysaccharides is that they are largely or entirely hexose polymers The nitrogen content may represent contamination or a structure containing an occasional hexosamine moiety

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with *C immitis* or *Sp schenkii* polysaccharide in the 50 µg /ml range, although these are optimal concentrations for the homologous systems

It is observed by complement fixation that the polysaccharides are not significantly more specific than the CFE's Although not tabulated, the crude protein preparations showed the least degree of specificity

No critical explanation is available to reconcile the apparent specificity of reactivity described for the agar plate double diffusion tests and the high degree of cross reactivity obtained with the same antibodies in complement fixation tests

The results suggest a greater degree of relationship among these dimorphic fungi and probably others, than is apparent from morphology

Skin Test Results

Results with the *B dermatitidis* and *H capsulatum* polysaccharide preparations injected into humans and infected guinea pigs have been reported [17] Additional experiments were conducted in which each volunteer was injected with polysaccharide and filtrate antigens The homologous polysaccharide preparations were indistinguishable in infected guinea pigs For this reason as well as for convenience one polysaccharide preparation of each organism has been employed

The polysaccharide preparations were all employed in a concentration of 10 µg in 0 1 ml saline injected intradermally and read at 24 48 and 72 hours The maximum reaction was taken for the final result In addition to the polysaccharides the volunteer group was injected with commercially available histoplasmin blastomycin and coccidioidin as well as a saline control In all therefore each individual received 8 injections, 4 on the volar surface of each forearm The sites for injection were chosen at random but each individual received the 8 injections at the same site In all 65 persons volunteered to be injected including the entire sophomore medical class and members of the staff of our different establishments No reactions were noted with saline or with polysaccharides from *Sp schenkii* or *B dermatitidis* commercial blastomycin yielded no reactions Results previously reported[17, 18] with *B dermatitidis* polysaccharide and blastomycin suggested somewhat greater sensitivity and lessened although not significantly so specificity of polysaccharide skin test material

Five individuals reacted to both histoplasmin and polysaccharide but as has been noted showed no cross reaction with the *B dermatitidis* reagents All 5 individuals had lived in the area of histoplasmosis endemicity in the middle western United States and in addition one had sustained a proved laboratory infection This latter individual showed the greatest sensitivity among the reactors

Five persons reacted to both coccidioidin and to *C immitis* polysaccharide 3 of the reactions showed less than 5 mm of induration All 5 persons had lived or spent time in the area of coccidioidomycosis endemicity No significant difference was noted in reactions to filtrate and polysaccharide antigens

It is apparent that these results do not resolve the problem of serologic and

Table 3

Complement Fixation Titers Rabbit Antibody versus CFE

Rabbit antibody dilutions	1	Cell free extract antigen diluted 1																			
		H capsulatum					*B dermatitidis*					*C immitis*					*Sp schenkii*				
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	40	4	4	4	4	0	4	2	0	0	0	4	4	3	2	0	3	2	0	0	0
	80	4	4	4	3	0	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	2	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
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	20	4	4	4	3	0	3	2	0	0	0	4	4	3	3	0	3	3	3	3	0
	40	3	2	2	2	0	3	2	0	0	0	4	3	3	1	0	2	2	2	2	0
	80	0	0	0	0	0	3	1	0	0	0	3	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
anti *C immitis*	10	4	4	4	4	3	4	1	0	0	3	4	4	4	4	2	4	4	4	4	2
	20	4	4	4	4	2	4	1	0	0	2	4	4	4	4	2	4	4	4	4	2
	40	3	3	3	2	0	4	1	0	0	0	4	4	4	4	0	3	0	0	0	0
	80	2	1	0	0	0	4	1	0	0	0	3	3	1	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
anti *Sp schenkii*	10	4	4	4	3	0	0	0	0	0	0	4	4	3	3	0	4	4	4	2	0
	20	4	4	4	3	0	0	0	0	0	0	4	4	3	3	0	3	2	0	0	0
	40	3	2	2	0	0	0	0	0	0	0	4	3	0	0	0	2	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Saline control, 0 no fixation, 4, complete fixation

with *C immitis* or *Sp schenkii* polysaccharide in the 50 µg /ml range, although these are optimal concentrations for the homologous systems

It is observed by complement fixation that the polysaccharides are not significantly more specific than the CFE's Although not tabulated, the crude protein preparations showed the least degree of specificity

No critical explanation is available to reconcile the apparent specificity of reactivity described for the agar plate double-diffusion tests and the high degree of cross reactivity obtained with the same antibodies in complement fixation tests

The results suggest a greater degree of relationship among these dimorphic fungi, and probably others, than is apparent from morphology

Skin Test Results

Results with the *B dermatitidis* and *H capsulatum* polysaccharide preparations injected into humans and infected guinea pigs have been reported[17] Additional experiments were conducted in which each volunteer was injected with polysaccharide and filtrate antigens The homologous polysaccharide preparations were indistinguishable in infected guinea pigs For this reason, as well as for convenience, one polysaccharide preparation of each organism has been employed

The polysaccharide preparations were all employed in a concentration of 10 µg in 0 1 ml saline injected intradermally and read at 24, 48, and 72 hours The maximum reaction was taken for the final result In addition to the polysaccharides, the volunteer group was injected with commercially available histoplasmin blastomycin and coccidioidin, as well as a saline control In all, therefore, each individual received 8 injections, 4 on the volar surface of each forearm The sites for injection were chosen at random, but each individual received the 8 injections at the same site In all 65 persons volunteered to be injected, including the entire sophomore medical class and members of the staff of our different establishments No reactions were noted with saline or with polysaccharides from *Sp schenkii* or *B dermatitidis*, commercial blastomycin yielded no reactions Results previously reported[17 18] with *B dermatitidis* polysaccharide and blastomycin suggested somewhat greater sensitivity and lessened, although not significantly so, specificity of polysaccharide skin test material

Five individuals reacted to both histoplasmin and polysaccharide but, as has been noted showed no cross reaction with the *B dermatitidis* reagents All 5 individuals had lived in the area of histoplasmosis endemicity in the middle western United States and, in addition, one had sustained a proved laboratory infection This latter individual showed the greatest sensitivity among the reactors

Five persons reacted to both coccidioidin and to *C immitis* polysaccharide, 3 of the reactions showed less than 5 mm of induration All 5 persons had lived or spent time in the area of coccidioidomycosis endemicity No significant difference was noted in reactions to filtrate and polysaccharide antigens

It is apparent that these results do not resolve the problem of serologic and

skin reactivity of polysaccharide materials derived from *B dermatitidis* or other systemic mycotic pathogens

In addition to other reasons previously mentioned for which the problem deserves continued consideration are the reports that repeated histoplasmin skin tests may result in the appearance of false positive reactions [18,19] Although it appears that specific polysaccharide may be employed as an immunizing agent against histoplasmosis in mice,[20] the amounts employed for skin testing may be less than the amount necessary to induce skin reactivity in humans If the opposite situation were to obtain, this would be good reason to abandon use of polysaccharide for skin tests

In summary, data are presented to suggest that strains of *B dermatitidis* vary quantitatively in mouse virulence and that, serologically, skin reactive polysaccharides can be prepared readily in large quantities from yeast phase broth of both virulent and avirulent strains

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Part IV Therapy

EXPERIENCE WITH AMPHOTERICIN B

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Amphotericin is an antifungal antibiotic produced by a streptomycete Its major antifungal activity *in vitro* is against those species having a yeastlike form in tissue TABLE 1 presents the range of *in vitro* sensitivity of selected human pathogens The values given are a composite of our own determinations and those in the literature [1-3]

We do sensitivity studies by incorporating the antifungal agent in melted Sabouraud s dextrose agar at *p*H 7 The dextrose agar has been accurately dispensed into 150 × 25 mm tubes prior to setting up the sensitivity studies The agar in the tubes is then melted and kept fluid in a water bath The desired amounts of antifungal agent are pipetted into the tubes of agar, well mixed and the tubes slanted and rapidly hardened in the refrigerator The surface of the slants is inoculated with 0 1 ml of a fungus suspension calculated to contain 20 to 100 infective particles Each series is set up in duplicate with duplicate controls The slants are incubated at 27° C

The inhibitory capacity of amphotericin B remains constant at room temperature for five days After this there is fairly rapid deterioration If easily recognized growth is not present in the control tubes by the fifth day the sensitivity studies will be inaccurate

We believe that grading and comparison of growth are easier on solid media than in liquids Furthermore our experience leads us to believe that there is a closer correlation between therapeutic effectiveness and *in vitro* sensitivity in solid media

Routes of Administration

The first clinical trials of amphotericin B were made with material for oral administration Relatively large doses produced therapeutic effects in some patients In others absorption of the antibiotic was entirely inadequate

Oral amphotericin was replaced by a suspension of the antibiotic in saline for parenteral use This material was insoluble was difficult to administer intravenously but was effective E R Squibb and Sons New Brunswick N J replaced this insoluble amphotericin B with a preparation that was combined with sodium deoxycholate buffered with sodium phosphate and lyophilized When water was added to this material a clear colloidal suspension was formed This preparation is spoken of here as solubilized amphotericin B We have represented it by the symbol S in our tables whereas the insoluble suspension is designated by the symbol I

In a previous report[4] we discussed the administration of solubilized amphotericin by intramuscular intravenous intrathecal and aerosol routes Bioassay and clinical observation suggested that the antibiotic was not absorbed from intramuscular sites Subsequent observations substantiate this view

Basic treatment is by the intravenous route We begin with a dose of 0 25

mg/kg of body weight The total dose for adults is increased by 5 mg every other day except in those patients with acute cryptococcal meningitis, for whom we increase dosage by 5 mg daily The maintenance dose is determined by toxicity and side effects, but is never greater than 1 5 mg/kg If side effects limit dosage to a level below 0 4 mg/kg of body weight, we either inject 25 mg of the sodium succinate ester of hydrocortisone intravenously at the time of starting the infusion or add it to the amphotericin glucose solution This has reduced the severity of side effects We routinely incorporate an antihistaminic in the infusion fluid

The calculated dose of amphotericin may be variously diluted and infused over a wide range of time, according to individual experience and prejudice We prefer to dilute the antibiotic with 500 ml of 5 per cent dextrose and administer it during a period of 2 or 3 hours Infants may be given the calculated dose in 50 to 100 ml of diluent

TABLE 1
MINIMAL INHIBITORY CONCENTRATION OF AMPHOTERICIN B FOR SELECTED PATHOGENIC FUNGI

Organism	Inhibitory range (µg/mL)
Blastomyces dermatitidis	0 05 to 0 5
Candida albicans	0 5 to 3 7
Cladosporium trichoides	1 0 (1 strain)
Coccidioides immitis	0 4 to 0 5
Cryptococcus neoformans	0 03 to 0 6
Histoplasma capsulatum	0 04 to 0 9
Sporotrichum schenckii	
Yeast phase	0 07 to 0 5
Mycelial phase	>40

Our assays have shown that solubilized amphotericin disappears from the blood stream rather rapidly during the first 2 postinfusion hours and at a much slower rate during the next 22 The 24-hour level usually has been between 0 5 and 1 0 mcg/ml of serum following the intravenous administration of between 0 6 and 0 8 mg/kg of body weight [4]

Since blood levels are well maintained and the yeastlike fungi are slow dividers many physicians choose to administer the antibiotic on alternate days We believe, without proof that daily treatment is more effective

We previously reported[4] that amphotericin B in its solubilized form is suitable for intrathecal administration We dissolve the material in water for injection, U S P, so that 1 0 mg of the antibiotic is contained in 0 5 ml of water Spinal fluid is withdrawn for laboratory studies, and 20 mg of the sodium succinate ester of hydrocortisone is then injected through the spinal needle A 5 cc syringe containing 1 0 mg of amphotericin in solution is connected to the spinal needle The syringe is filled with spinal fluid and 1 0 ml slowly reinjected The syringe is refilled with spinal fluid and another 1 0 ml is injected The amphotericin is diluted by spinal fluid in this manner 4 or 5 times before completely emptying the syringe

Whenever possible, we inject amphotericin intrathecally in the sitting position. Immediately following injection, the patient is asked to lie on his back for fifteen minutes, and then to assume the prone position. We restrict intrathecal injection to twice each week.

We have not detected amphotericin in the spinal fluid during the intravenous treatment of any patient whose spinal fluid protein was less than 75 mg per cent. We have reported that assayable amounts of the antibiotic diffuse into the spinal fluid when the protein level is high.[4] Intrathecally injected amphotericin was not detected in the spinal fluid beyond 48 hours.

The relatively low concentrations of amphotericin in the spinal fluid of patients with central nervous system cryptococcosis undergoing treatment intravenously are apparently adequate for sterilization in patients with very susceptible organisms. The highest concentration found was 0.46 µg/ml in a spinal fluid whose protein was 450 mg per cent. Multiple assays on 3 patients with cryptococcal meningitis indicated that one can anticipate concentrations of amphotericin between 0.17 and 0.27 µg/ml if the spinal fluid protein is above 150 mg per cent and intravenous dosage is at least 0.33 mg of solubilized antibiotic per kilogram of body weight per day.

Since the *in vitro* sensitivity of the yeastlike fungi varies widely, we believe it desirable to give amphotericin intrathecally early in the treatment of mycotic meningitides. Winn[3] believes this procedure should be followed in all cases of coccidioidal meningitis.

Both the insoluble suspension and the solubilized antibiotic were well tolerated when injected into abscesses, pyoarthroses, suppurated lymph nodes, areas of osteomyelitis, and pleural empyema. Up to 30 mg of the solubilized antibiotic was required to sterilize a coccidioidal pyoarthrosis. This amount produced no side effects.

Results of Treatment

Patients 1 through 21 were the basis for a report given in June 1958.[4] They have remained under observation and are included in the tables and text that follow, retaining the original numerical designation. Patients 22 and 23 were discussed in our previous report, but were not directly under our care and are omitted from the present report. Patients 16, 6, and 8 have been re-treated since our 1958 report. Of these, only patient 6 represents re-treatment for relapse. Patient 8 had received only intramuscular amphotericin when first reported. A second course of treatment was given to patient 16 subsequent to our first report. Original treatment had been interrupted because of azotemia, and the second course was administered only because of our conviction that the first course was inadequate for treatment of acute cryptococcal meningitis.

Patients 24 through 32 have been treated since June 1958. It is our policy to maintain follow-up observation of our patients at intervals no greater than 3 months during the first year and 6 months thereafter.

Toxicity and Side Effects

Intravenous administration of amphotericin B is accompanied by a high incidence of side effects and significant toxicity The reactions noted by us in the treatment of 29 patients are listed in order of frequency in TABLE 2

Reactive fever occurs during or following infusion in most patients It is usually moderate and persists until maintenance dosage is reached Continued daily administration of the same dose is accompanied by reduction or disappearance of reactive fever We believe the pyrogenic activity of amphotericin B is in some way related to impurities rather than the antibiotic itself

TABLE 2
TOXICITY AND SIDE EFFECTS OF INTRAVENOUS AMPHOTERICIN B

Type of reaction	Proportion of patients having reaction	Comment
Fever	27/29	One patient had fever once
Chills	25/29	One patient had single chill
Anorexia	16/29	Always at high dosage
Nitrogen retention	13/29	
Headache	12/29	During infusion at high dosage
Abdominal pain	10/29	Predominantly epigastric
Nausea	9/29	Unrelated to azotemia
Vomiting	6/29	
Phlebitis	4/29	
Melena	1/29	No ulcer demonstrated
Hemorrhagic gastroenteritis	1/29	At autopsy
Blurring of vision	1/29	
Exfoliative dermatitis, Purpura, Convulsions, Death	1/29	All in 1 infant at high dosage
Muscular weakness, Polymyositis	1/29	
Chest pain, Bradycardia with dropped beats, Palpitation	1/29	Only with refrigerated infusion fluid
Hematemesis, Probable duodenal ulcer	1/29	Asymptomatic when treatment discontinued

This opinion is based on the repeated observation of rigors and moderate to severe fever when a preparation bearing a new lot number has been administered to patients who have achieved maintenance dosage

Chills, fever, headache, and nausea are usually kept at a tolerable level by premedicating the patient with acetylsalicylic acid and incorporating an antihistaminic in the infusion fluid If these measures fail, 25 mg of the sodium succinate ester of hydrocortisone given intravenously has been remarkably effective in our experience

Anorexia has been a serious problem in three patients and a significant complaint in slightly more than one half of those treated intravenously In only a few patients has this been accompanied by weight loss Anorexia is usually lessened by hydrocortisone

Abdominal pain during intravenous infusion has been frequent but rarely severe. The pain is predominantly epigastric, cramping in nature and is irregularly modified by anticholinergics and antispasmodics. One patient in whom no peptic ulcer could be demonstrated, had abdominal pain and melena to a degree requiring transfusions. One patient died from acute staphylococcal pneumonia on the day following the last infusion of amphotericin. This patient had complained of severe epigastric pain following each infusion. Autopsy revealed a superficial hemorrhagic gastroenteritis that was quite similar to that described in dogs treated with intravenous amphotericin.[6] However similar pathology may result from severe staphylococcal infection. A third patient with persistent epigastric pain had two small hematemeses. Gastrointestinal X rays showed a deformity of the duodenal bulb with probable ulceration. This patient had no previous history of gastrointestinal complaints and her symptoms disappeared promptly when amphotericin was discontinued.

Vomiting occurred infrequently in our experience. It became persistent in two patients who also had severe anorexia. One of these developed profound muscular weakness, albuminuria and polymyositis proved by biopsy. We initially suspected this to be due to hypersensitivity to amphotericin B. However hypokalemia was found and the patient's symptoms disappeared dramatically following infusions of potassium chloride solution. A patient currently under treatment, not included in TABLE 2, developed a similar picture. Weakness became so great that he could not raise his head from the pillow. Serum electrolytes were within normal range but the electrocardiogram was typical of hypokalemia. Intravenously administered potassium restored his muscular strength within a few hours.

Phlebitis has occurred infrequently in our patients in contrast to the high incidence observed by some other physicians. A needle left within the vein for many hours is a recognized cause of phlebitis. Acidic solutions may irritate veins. We have found that some commercially prepared 5 per cent glucose solutions have a pH of less than 5.0 at the time they are used for infusions. Our low incidence of phlebitis may be due in part to the relatively short duration of our infusions and in part to the use of 5 per cent glucose solutions near neutrality.

Azotemia has been the most important toxic manifestation of intravenous amphotericin B in so far as the regulation of dosage is concerned. In our experience, decreased urea clearance was unaccompanied by albuminuria, cylindruria or abnormal microscopic hematuria in most patients. Azotemia did not develop in either patient 14 or patient 17 who received the largest doses of the solubilized antibiotic on a body weight basis.

Patient 32 developed nitrogen retention at a dosage of 0.26 mg./kg. of body weight and his blood urea nitrogen remained above 30 mg. per cent on a daily dose of 0.3 mg./kg. of body weight. This patient had normal urinalysis and blood chemistries prior to treatment.

Patient 16 developed persistent azotemia on a daily dose of 0.7 mg. of the solubilized antibiotic per kilogram of body weight during his first course of treatment. Treatment was discontinued due to azotemia. We attempted to

reinstitute intravenous therapy 2 months later The fourth infusion contained 0 38 mg of amphotericin per kilogram of body weight and produced elevation of the blood urea nitrogen to 24 mg per cent Intravenous amphotericin was discontinued Sixteen months later his blood urea nitrogen was still 28 mg per cent, although his blood creatinine was only 1 31 mg per cent and his urinalysis normal Other studies showed a urea clearance of 26 2 ml /min but a nonurea osmolar clearance that was within normal limits This is the only patient in our group who has shown evidence of renal damage of a probably permanent type

During treatment of our first 19 patients we reduced the daily dose of amphotericin whenever the blood urea nitrogen rose above 20 mg per cent We have paid less attention to this toxic reaction in managing our last 10 patients If nitrogen retention develops at a dose below 0 5 mg /kg of body weight per day we continue treatment at the same or a higher dosage so long as the blood urea nitrogen does not rise above 50 mg per cent

Only 2 infants were among our patients Patient 27 had severe rigors with concomitant cyanosis until maintenance dose was reached The daily dose was increased rapidly due to the serious nature of his illness The patient also had frequent vomiting Patient 21 became cyanotic during severe rigors The last two infusions were accompanied by brief convulsions When exfoliative dermatitis and purpura appeared treatment was stopped We believe this infant s death was in part due to amphotericin toxicity

Some physicians have expressed the opinion during informal conferences that refrigeration of the amphotericin glucose solution prior to infusion reduces its toxicity and side effects Refrigeration for 24 48 and 72 hours has been suggested We have attempted to evaluate this in patients who have developed azotemia or persistently troublesome side effects by changing from freshly prepared solutions to refrigerated ones Treatment of 2 patients was initiated with refrigerated solutions and changed to freshly prepared ones when the same indications arose All refrigerated solutions were brought to room temperature prior to infusion We have been unable to detect any difference in the toxicity or acceptability of the 2 types of solutions

Patient 28 developed a sharp left lateral chest pain that radiated substernally during infusion of refrigerated amphotericin solutions Bradycardia and occasional dropped beats were present at such times Minor alterations in the T waves of the electrocardiogram were present in precordial leads 4 5 and 6 Both findings and complaints disappeared when a freshly prepared solution of amphotericin was substituted for refrigerated material

Intrathecal amphotericin B has been free from serious toxic reactions when given in the manner and dosage previously described Paresthesia developed regularly within a few minutes after intrathecal instillation When the antibiotic was instilled in the sitting position these paresthesias were usually confined to the area below the waist Transient paresis of one or both lower extremities has occurred on several occasions but has never persisted beyond 72 hours Catheterization for urinary retention during the first 24 hours following intrathecal administration has been necessary on two occasions Temporary mild relaxation of the anal sphincter occurred in one patient

Patient 30 developed right sciatic pain in addition to mild paresis of the right leg following the tenth intrathecal dose No further intrathecal treatments were given Without suggestions or questioning this patient continued to complain of right sciatic pain during the first hour or two of each subsequent intravenous infusion of amphotericin The pain did not persist between infusions We were particularly interested in this phenomenon since it suggested that sufficient amphotericin was diffusing into the spinal fluid to produce pain in the distribution of the already damaged roots The spinal fluid protein exceeded 300 mg per cent at the time

Treatment of Blastomycosis

Seven patients with systemic blastomycosis have been treated with amphotericin B The antibiotic was given initially by the intramuscular route to 2 patients We observed no effect in one who had both cutaneous and pulmonary lesions The second patient 8 is discussed later The essential clinical data are in TABLE 3

Intravenous amphotericin B produced prompt and unequivocal response in every patient Patient 5 succumbed to a nosocomial staphylococcal pneumonia just prior to the planned termination of treatment There was no histological or cultural evidence of persisting blastomycosis at autopsy Patients 7 9 28 and 29 healed rapidly and have remained well

Patient 6 had disease that recurred following each course of treatment with iodides diamidines oral amphotericin and short courses of intravenous amphotericin B In a previous report [4] we stated that he healed completely following 90 daily infusions of amphotericin However relapse occurred 6 months later The organism isolated during this exacerbation had the same *in vitro* sensitivity to amphotericin B as before Retreatment was given daily for 130 days and then interrupted to start plastic reconstruction of his nose The surgically removed tissue was negative histologically and culturally for *Blastomyces dermatitidis* Two months later treatment was resumed for an additional 38 days making a total of 168 days of treatment The patient remained well during the subsequent 10 months

Treatment of patient 8 is worthy of comment This patient was transferred to us following diagnosis by resection of the upper lobe of the right lung Forty three daily intramuscular injections of amphotericin B were given prior to discharge The disease became active 4 months later Extensive cavitation in the remaining lobes of the right lung was present at readmission

The initial infusions of amphotericin B were accompanied by unusually severe reactions despite a low dose of 10 mg Various antiemetics and antispasmodics had little effect upon nausea and vomiting Treatment on alternate days did not lessen the reactions Azotemia developed and was accompanied by albuminuria The spinal fluid protein was 76 mg per cent When cutaneous hyperalgesia appeared followed by profound muscular weakness hypersensitivity vasculitis was suspected Amphotericin was discontinued after only 42 days of treatment

Muscle biopsy demonstrated patchy areas of degeneration with adjacent

sarcolemmal proliferation Hypokalemia was found The patient s symptoms disappeared promptly after treatment with potassium intravenously She regained her weight and sense of well being after one month without any treatment Her sputum was negative for *B dermatitidis* by direct examination culture, and animal inoculation Nevertheless, it was decided that additional therapy was desirable

TABLE 3
TREATMENT OF BLASTOMYCOSIS WITH AMPHOTERICIN B*

Patient	Age (yr)	Clinical status before treatment	Duration of treatment, days	Average daily dosage mg kg	Response and follow up
5	76	Disseminated moribund	26 I M	1 0 (S)	None
			58 I V	0 65 (S)	Promptly improved Death during R_x from staphylococcal pneumonia
6	46	Disseminated cachectic	6 I V	0 92 (I)	Good relapse
			12 I V	0 46 (I)	Improved
			8 I V	0 92 (I)	Improved
			6 I V	1 4 (I)	Relapsed
			90 I V	0 62 (S)	Healed relapsed 6 mo later
			168 I V	0 52 (S)	Healed Well 10 mo p̄ R_x
7	57	Disseminated cachectic	78 I V	1 25 (I)	Healed Well 36 mo p̄ R_x
8	37	Involvement of mediastinum & questionably liver ambulatory	43 I M	0 7 (S)	Progressive disease 4 mo p R_x
		Pneumonitis with cavitation & med astinitis	46 I V	0 42 (S)	R_x stopped due to toxicity Healed after diamidines and surgery Well 3 mo p R_x
9	69	Disseminated severely ill,	62 I V	0 43 (S)	Healed Well 28 mo p R_x
28	35	Good Involvement of bone & soft tissues	67 I V	0 54 (S)	Healed Well 5 mo p R_x
29	31	Good Pulmonary pharyngeal laryngeal involvement	90 I V	0 54 (S)	Healed Well 13 mo p̄ R_x

* The following abbreviations are used in TABLES 3 to 6 inclusive I M intramuscular I V intravenous I T 1 mg amphotericin B intrathecally (I) suspension of insoluble amphotericin B (S) solubilized amphotericin B p̄ after q every o other d day R treatment

It was necessary to interrupt treatment with intravenous hydroxystilbamidine on the fourteenth day due to the appearance of pneumococcal infection with bacteremia After appropriate treatment for the pneumococcal infection hydroxystilbamidine was reinstituted daily for 1 month and on alternate days for an additional 6 weeks

Although her sputum remained negative for *Blastomyces* it was decided to resect the remaining lobes of the right lung because of persistent cavitation Mediastinal and hilar lymph nodes were greatly enlarged, but so matted they could not be removed safely The lung contained abscesses with areas of necrosis and granulation tissue Atypical foreign body giant cells were present

but no fungi were seen by special staining. Cultures of the resected lung were negative for *B. dermatitidis*.

In summary, of the 7 patients treated intravenously with amphotericin B 5 were healed, 1 was mycologically negative at the time treatment was changed to hydroxystilbamidine, and 1 died from an unrelated cause.

Treatment of Cryptococcosis

Eight patients with central nervous system cryptococcosis are included in TABLE 4. Patients 10 and 15 should not be considered in evaluating the effectiveness of amphotericin B. Patient 10 was moribund at the time of admission and died during the fourth day of treatment. Patient 15 showed obvious regression of cutaneous lesions during 14 days of intravenous therapy. He refused further infusions, but did accept 26 days of oral medication during which the cutaneous lesions rapidly worsened. He died 6 months later.

Of the 6 patients who completed the planned course of treatment, none has relapsed. Three of these have been followed closely for more than 2 years. The spinal fluid of all 6 is sterile, acellular, and contains normal concentrations of dextrose and chloride. The protein is normal in patients 14 and 16 who received both intravenous and intrathecal treatment. Cultures of the spinal fluid of patient 13 were negative following 14 intrathecal injections of amphotericin given during the course of intramuscular treatment. After we had determined that intramuscular treatment was ineffectual the patient was recalled for intravenous treatment. The spinal fluid protein remains elevated in this patient. Patient 30 is still convalescing but the other 5 are pursuing their regular occupations or household duties without restriction or symptoms.

No spinal fluid specimen was positive culturally for *Cryptococcus neoformans* after the fourteenth day of treatment. Cryptococci could be found microscopically beyond this time in 2 patients. We tried unsuccessfully to prove their viability. The organisms were separated rapidly from the spinal fluid by centrifugation. Growth of *C. neoformans* was not found in either solid or liquid media even after weeks of incubation.

TABLE 4 shows that we have depended primarily on daily intravenous treatment. It was supplemented by intrathecal administration in 4 patients. Three of these had a cerebrospinal fluid protein level below 100 mg per cent at the time treatment was started. The total duration of all treatment was 4 months or more in 5 of the 6 patients who completed their planned course of therapy.

Because of inability to demonstrate amphotericin B in the spinal fluid of intravenously treated patients whose spinal fluid proteins were below 78 mg per cent, we shall continue to use semiweekly intrathecal antibiotic during the first months of treatment. We have no proof that diffusion of assayable amounts of amphotericin into spinal fluid is necessary for successful therapy. In addition, one might reasonably doubt that amphotericin instilled into the spinal fluid would diffuse into parenchymal cysts located at a distance from the ventricular system.

Relatively speaking we have had extensive experience with central nervous system cryptococcosis. Prior to the use of amphotericin, all of our patients

Table 4

Treatment of Cryptococcosis with Amphotericin B*

Patient	Age (yr)	Clinical state before treatment	Involvement	Treatment	Average daily dosage mg/kg	Clinical response & follow up
10	67	Comatose moribund	CNS adrenals kidneys	4 IV	1 66 (I)	Died on 4th day of R_x
11	43	Semicomatose delerious poor	CNS	115 IV 96 IV	1 07 (I) 0 35 (S)	Working full time Well 32 mo p R_x
12	52	Comatose poor	CNS	20 IV 88 IV 74 IV (q o d) IT (14)	1 0 (I) 0 54 (S) 0 54 (S)	Full activity Well 30 mo p R_x
13	63	Ambulatory good	CNS	90 IV IT (2)	0 57 (S)	Well 20 mo p̄ R_x
14	34	Good ambulatory	CNS	56 IV IT (11)	1 0 (S)	Full activity Well 27 mo p R_x
15	72	Fair ambulatory	Skin bones liver lungs spleen CNS	14 IV 26 oral	0 75 (I) 50	Improved during IV R_x rapid relapse during oral R_x Died 6 mo p R_x
16	15	Acutely ill delirious.	CNS	59 IV IT (17) 51 IV IT (10)	0 70 (S) 0 29 (S)	Attending college well 18 mo p 2nd R_x
30	51	Good febrile	CNS	120 IV IT (10)	0 45 (S)	Well 1 mo p R_x

* See TABLE 3 for key to abbreviations

died from their infection. Observation of patients treated with amphotericin B has been continued for a sufficient time to permit cautious enthusiasm.

We suspect that treatment of central nervous system cryptococcosis with amphotericin will follow the same errant pathway as the antimicrobial treatment of tuberculous meningitis. Because of the nature of the pathology produced by *Cryptococcus* in the central nervous system, relapse may not occur for months or years after apparently successful treatment. This makes it difficult to determine the optimal duration of treatment. Infections of the meninges without deep seated parenchymal cysts may be cured by no more than two months of treatment. Four months of treatment may be inadequate for more deeply seated lesions.

Treatment of Histoplasmosis

We have completed the treatment of 8 patients with histoplasmosis. Seven of these exhibited clinical features that Furcolow[7] associates with a guarded prognosis. The essential therapeutic data are in TABLE 5.

Patient 1 had primary disease of the type that is usually self limited. Illness was relatively prolonged and appeared unaltered by oral amphotericin. Patients 2 and 3 received oral amphotericin in addition to the intravenous medication. The orally administered antibiotic has been omitted from TABLE 5.

Patient 3 had chronic disseminated disease of the progressive type. Response to treatment was excellent and rapid. He remained well for 15 months at which time he developed a recurrent lesion of the maxillary alveolar ridge. Retreatment was terminated after 3 days due to the appearance of cyanosis and severe hypotension. Clinically, the patient's death 24 hours later was ascribed to an anaphylactic reaction to amphotericin.

Autopsy revealed infarction of the distal ileum with perforation and generalized purulent peritonitis. This patient had experienced vascular occlusive episodes in the past without relation to amphotericin B therapy. Both adrenals were greatly enlarged. They contained both acute granulomatous lesions associated with *Histoplasma* and areas of caseation and fibrosis devoid of organisms. The granulomatous areas were culturally positive for *Histoplasma capsulatum*. Active histoplasmosis was found in the adrenals and gingival ulcer only. The autopsy studies supported our beliefs that *Histoplasma* persisted in the adrenals and that the initial course of treatment was inadequate.

Response of patient 27 is more difficult to interpret. This infant was desperately ill at the time of admission. He had suffered one month of persistent diarrhea, progressive anemia, chills and fever to 105° F daily. Cyanosis was observed during rigors. The liver and spleen were greatly enlarged and bilateral central pneumonitis was seen by X ray. *H. capsulatum* was seen in stained smears of bone marrow and recovered in culture. Intravenous treatment was given daily between September 19, 1958 and November 14, 1958. The prompt clinical response and the difficulties created by daily intravenous infusion of a small infant led us to treat him on alternate days between November 14, 1958 and February 6, 1959. At this time, the chest X ray was normal, physical examination revealed no enlargement of the liver or spleen, and the patient was discharged.

One month later he contracted a middle ear infection with fever and anorexia A pediatrician was consulted In addition to the middle ear disease, he found enlargement of the liver and spleen Aspiration of the bone marrow revealed intracellular *H capsulatum* in the stained smear Unfortunately the marrow was not cultured The infant appeared to recover completely during a three and one half month's course of triple sulfonamides The pediatrician considered him normal two months after treatment was stopped The patient was lost to follow up

TABLE 5
TREATMENT OF HISTOPLASMOSIS WITH AMPHOTERICIN B*

Patient	Age	Classification of disease	Duration of treatment days	Average daily dosage mg kg	Response and follow up
1	7 yr	Moderately severe primary	164 oral	90	No definite change in course Recovered Well 29 mo p R_x
2	32 yr	Reinfection, moderate	15 I V 15 I V (q 3d)	1 25 (I) 1 25 (I)	Prompt improvement Prompt improvement
3	58 yr	Chronic progressive	86 I V 70 I V	0 37 (S) 0 87 (I)	Healed Well 30 mo $\bar{p}$ R_x Prompt healing Relapse 15 mo p R_x
4	45 yr	Chronic progressive	8 I V 79 I V	0 57 (S) 0 63 (S)	Healed Well 23 mo p R_x
24	59 yr	Chronic progressive	91 I V	0 66 (S)	Healed Well 11 mo $\bar{p}$ R_x
25	64 yr	Chronic progressive	90 I V	0 58 (S)	Prompt improvement sputum conversion Well 2 mo p R_x MRD 112† concurrently
26	48 yr	Chronic progressive	61 I V	0 5 (S)	Healed Well 11 mo p R_x
27	11 mo	Progressive disseminated	57 I V. 42 I V (q o d)	0 88 (S) 1 0 (S)	Dramatic response R_x inadequate apparent recovery after sulfonamide Well 10 mo p R_x

* See TABLE 3 for key to abbreviations
† MRD 112 is β-diethyl aminoethyl fencholate

It is known that intracellular *H capsulatum* may be seen in lesions from which they cannot be recovered culturally or by animal inoculation Nevertheless, the clinical course of this infant suggests that histoplasmosis was still active after four and one half months of intravenous treatment

Patients 24 and 26 had progressive ulcerative and granulomatous mucous membrane lesions with visceral dissemination Healing was prompt, and both have remained well

The type of histoplasmosis present in patients 4 and 25 is difficult to evaluate therapeutically without prolonged observation These patients had chronic progressive pulmonary disease with extensive cavitation The constitutional manifestations of disease subsided promptly during treatment with amphotericin, and *H capsulatum* disappeared from the sputum Chest X rays revealed clearing of the exudative disease but persistence of fibrosis and cavitation The problem is similar to that in chronic cavitary tuberculosis

The left upper lobe was resected from patient 4 at the termination of treatment with amphotericin. The wall of the cavity consisted of a thin layer of fibrous connective tissue without any evidence of inflammation in its wall or the adjacent lung. Granulomas within the lung were well encapsulated, showed caseation necrosis centrally, and were negative for *H. capsulatum* and mycobacteria by special stains. One half of one of the larger nodules was triturated. Portions of this material were sterile culturally and by animal inoculation.

Studies of the resected lobe indicate that the results of amphotericin treatment of this patient with chronic cavitary histoplasmosis were similar to those achieved by isoniazid and paraminosalicylic acid in some patients with chronic cavitary tuberculosis. If amphotericin B can bring about the so called "open negative" status (persistent cavitation with culturally negative sputum) in the majority of patients with chronic cavitary histoplasmosis, it will represent a major therapeutic advance. Many of these patients are not suitable for surgical treatment. As with isoniazid in relation to cavitary tuberculosis, the rate of relapse from the open negative state can be determined only when a statistically significant population has been followed for a minimum of three years.

In our experience, the initial response of histoplasmosis to amphotericin B has been similar to that of tuberculosis when treated with either streptomycin or isoniazid alone. Microbial persistence and relapse have occurred. The sensitivity to amphotericin B of the *H. capsulatum* isolated from patient 3 at autopsy was the same as that determined from the pretreatment culture.

We believe that the optimal duration of treatment needs to be established and the possibilities of combined chemotherapy investigated. The latter consideration led us to treat patient 25 with amphotericin B intravenously and beta diethylaminoethyl fencholate orally. The oral drug has been tolerated well in large doses, and *in vitro*[9] studies have indicated that beta-diethylaminoethyl fencholate has a mycostatic effect.

Treatment of Coccidioidomycosis

Our experience with coccidioidomycosis is too inadequate to be very meaningful. The two patients who have completed therapy are included in TABLE 6. Patient 17 had severely disseminated disease. Cutaneous anergy to coccidioidin was present throughout her illness, and complement fixing antibodies against coccidioidin were present in serum diluted 1:256. The patient received more than 12 gm. of the insoluble suspension of amphotericin intravenously and almost 7 gm. of the solubilized antibiotic. She died from coccidioidomycosis during the course of treatment.

Patient 31 developed infiltration in her right lung some time between 1940 and 1944. She was discharged without diagnosis after 9 months in a sanatorium in 1944. Between 1946 and 1956 she had recurrent "bronchitis" during which she would raise small amounts of blood flecked sputum. The area of disease in her right lung increased in early 1956, and new disease appeared in the left lung. Chemotherapy for tuberculosis was begun in 1956 and continued until 1959. The patient was hospitalized briefly in 1956 following an hemop-

TABLE 6
TREATMENT OF MISCELLANEOUS DEEP MYCOSES WITH AMPHOTERICIN B*

Patient	Age	Diagnosis	Clinical status before treatment	Treatment days	Average daily dosage mg./kg	Response & follow up
17	35 yr	Coccidioidomycosis disseminated	Moribund	66 I V } 20 oral } 113 I V 65 I V 88 I V 48 I V (q o d) Course 2† 18 I V 65 I V 34 I V	3 0 (I) 242 0 1 5 (I) 0 6 (S) 0 43 (S) 0 43 (S) 0 6 (S) 1 0 (S) 1 14 (S)	Remarkable improvement, became ambulatory Progression of disease during lower dosage Temporary improvement, death
31	45 yr	Coccidioidomycosis disseminated	Good	95 I V 46 I V (q o d)	0 52 (S) 0 58 (S)	Sputum conversion and excellent serologic response Hydroxystilbamidine concurrently Well 2 mo p Rx
18	61 yr	Sporotrichosis disseminated	Cachectic severely impaired gait	58 I V	0 63 (S)	Healed Normal gait Well 22 mo p Rx
19	30 yr	Sporotrichosis lymphocutaneous	Good	49 I V	0 42 (S)	Healed Well 20 mo p Rx
32	22 yr	Sporotrichosis lymphocutaneous	Good	30 I V	0 30 (S)	Healed Well 11 mo p Rx
20	62 yr	Moniliasis (pyelonephritis)	Poor uremic	6 I V	0 25 (S)	Became afebrile with negative blood and urine cultures Death from cardiac failure
21	6 mo	Moniliasis	Poor congenital agammaglobulinemia	13 I V 13 I V	2 0 (I) 3 0 (I)	Healing of pulmonary and hepatic involvement died

*See TABLE 3 for key to abbreviations
† Course 2 was separated from Course 1 by 38 days at home and treatment with intravenous culcin between September 3 and October 31 1957

tysis During this time numerous sputa and gastric washings were studied without finding any pathogenic organisms Skin tests with histoplasmin coccidioidin blastomycin and 1 100 old tuberculin were negative

This patient was rehospitalized in 1957 because of persistent cavitary disease A right upper lobectomy was performed A diagnosis of coccidioidomycosis was made from histopathological study of the resected lobe The coccidioidin skin test was again negative but complement fixing antibodies were present against coccidioidin in a titer of 1 64

Multiple areas of cavitation appeared in the right lower lobe during late 1958 and early 1959 The patient s sputum became positive for *Coccidioides immitis* and she was referred to us for treatment The coccidioidin complement fixation titer was 1 128 and the coccidioidin skin test was negative prior to treatment The patient received 3 775 gm of amphotericin B and 9 225 gm of hydroxystilbamidine intravenously Sputum conversion was obtained promptly and the complement fixation titer fell from 1 128 to 1 8 within 6 months The immediate response of this patient has been excellent but follow up is too brief to justify optimism

A patient with coccidioidal meningitis is currently under treatment The initial response has been excellent and intrathecal therapy well tolerated William A Winn[5] has had significant experience with coccidioidomycosis and believes that many such patients can be successfully treated with amphotericin B properly administered

On the basis of our observations of patient 17, both during life and at autopsy we believe that amphotericin is unlikely to be successful in patients with severely disseminated coccidioidomycosis who have large visceral abscesses that are inaccessible to surgical drainage or repeated instillation of amphotericin

The course of patient 31 exemplifies the well recognized hazard of resection for pulmonary coccidioidomycosis New cavitary disease develops within the unresected lung with disturbing frequency It is desirable to perform necessary surgery for coccidioidomycosis during the course of intravenous treatment with amphotericin B Winn recommends 3 or 4 months of treatment[5]

Treatment of Sporotrichosis

One patient with disseminated sporotrichosis and 2 with lymphocutaneous disease have been treated with amphotericin B They are included in TABLE 6 All have healed without relapse to date Patient 18 with disseminated disease has been previously reported Two months of intravenous therapy seemed adequate to control his disease which involved the pharynx larynx lungs bones lymph nodes and cutaneous tissues Patient 32 was treated with relatively small doses of amphotericin for only 30 days Our purpose was to determine whether relapse would occur The patient has remained healed for 11 months

We do not recommend treatment of lymphocutaneous sporotrichosis with intravenous amphotericin This type of disease responds well to oral iodides in most instances Patients who are sensitive to iodides or who fail to respond well to the medication can be treated with hydroxystilbamidine or amphotericin We believe that hydroxystilbamidine is easier and safer to administer

Infiltration of the lesions of lymphocutaneous sporotrichosis with a solution of amphotericin B in procaine is entirely feasible. It would be interesting to combine a short course of oral iodides with locally injected amphotericin.

Treatment of Moniliasis

Neither of the two patients under our direct management for monilia had disease suitable for therapeutic evaluation. Patient 20 had cardiorenal disease, which was responsible for his death after only 6 days of treatment with intravenous amphotericin. Blood and urine cultures which had been positive for *Candida albicans*, became sterile during therapy. The patient became afebrile within 24 hours after the first infusion.

Patient 21 was an infant with congenital agammaglobulinemia who probably developed candidiasis as a complication of antibiotic treatment for staphylococcal infection. Treatment with amphotericin controlled his pulmonary moniliasis and sterilized his blood stream. Death was probably due in large part, to amphotericin. Cultures of the lung and multiple minute abscesses in the liver were negative at autopsy for *Candida*. It is not known whether the abscesses of the liver were produced by *Candida* or by the prior-existing staphylococcal disease.

Our experience permits no estimate of the value of amphotericin B for the treatment of systemic candidiasis. In the past we have observed several patients with positive blood and urine cultures for *C. albicans* during the course of prolonged therapy with broad spectrum antibiotics. Blood and urine cultures became negative, and the patients recovered after antibiotics were discontinued. No antifungals were given. We believe that this disease is difficult to evaluate therapeutically unless definite tissue invasion can be demonstrated.

Discussion and Summary

Amphotericin B is an initially effective antibiotic for the treatment of blastomycosis, cryptococcosis, histoplasmosis and sporotrichosis. Post treatment follow up has been long enough to indicate that the initial good results are maintained in a high percentage of those receiving intravenous treatment for two or more months. Amphotericin should be valuable in all fungus infections having a yeastlike tissue phase. We have had insufficient experience to form an opinion of its effects on systemic candidiasis and coccidioidomycosis.

In its present form, this antibiotic is suitable for intravenous, intrathecal, and local instillation. Intramuscular injection produces neither detectable blood levels nor healing of susceptible infections. Amphotericin B is poorly and irregularly absorbed from the alimentary tract.

We have treated 29 patients with amphotericin B by the intravenous route. There have been 2 relapses and 4 treatment failures. Two of the failures can be eliminated in an attempt to evaluate the efficacy of intravenous amphotericin. One of the failures died after only 4 infusions and the second refused intravenous therapy after the fourteenth day. Both had cryptococcosis with central nervous system involvement.

Analysis of the 2 real failures and the 2 relapses does not lead to any conclusions that, if heeded, might prevent future failure. Patient 17 and, perhaps,

patient 27 never achieved initial healing. Patient 17 responded dramatically during early daily treatment with the insoluble form of the antibiotic. The most obvious deterioration occurred during alternate day treatment with 0.43 mg of the solubilized antibiotic per kilogram of body weight. The second course of therapy was given daily with a dosage range between 0.6 and 1.14 mg/kg of body weight. The sensitivity of the infecting *C. immitis* was unchanged throughout treatment. The total duration of treatment was 16 months. The most obvious cause for failure of treatment was the presence of multiple large abscesses that were inaccessible to surgical drainage or direct instillation of amphotericin. These abscesses contained viable *C. immitis* and were the probable source for repeated hematogenous dissemination.

Patient 27, an infant, was treated for 57 days at a level of 0.88 mg/kg of body weight, and this was followed without interruption by 42 infusions on alternate days at a level of 1.0 mg/kg. *H. capsulatum* was seen in his bone marrow within a month after termination of treatment. One may suspect that either the total duration of treatment (four and one half months) was inadequate or that alternate-day treatment was the principal cause of failure. Both alternatives represent only the obvious and perhaps superficial possibilities.

Patients 3 and 6 relapsed. Patient 3 received the insoluble suspension of amphotericin for all but eight of his infusions. As we have previously reported,[4] a variable amount of the insoluble suspension of amphotericin in the infusion fluid actually reached the patient's blood stream. The true daily dose was always conjectural. The total duration of intravenous therapy was only 78 days, which is probably inadequate for disseminated histoplasmosis.

Recurrence in patient 6 took place 6 months after 90 daily infusions at an average dose of 0.62 mg/kg of body weight. The infecting organism *B. dermatitidis* was inhibited completely by 0.06 μg of amphotericin per ml of Sabouraud's dextrose agar. The sensitivity of the organism under the same conditions had not changed when it was redetermined during relapse. Serum assays of amphotericin following infusions of the average maintenance dose showed a level of 1.0 μg/ml or higher during the first 4 hours after infusion and a level of 0.5 μg/ml 24 hours after infusion. In our opinion this indicates adequate dosage. The fact that this patient's disease had reappeared many times after treatment with various drugs may be pertinent. However, his past history did not indicate unusual susceptibility to other infections. Retreatment for 168 days at a smaller daily dose has been successful to date.

We draw no specific conclusions from the small group of failures. It seems obvious that optimal dosage and duration of treatment for the various mycoses are still unknown. Comparison of our treatment protocols with those of others makes us believe that we are using a smaller daily dose for longer periods of time. We have made no conscious attempt to lower dosage, striving to achieve a range between 0.5 and 0.8 mg/kg of body weight of the solubilized suspension per day. That we have not always achieved this range is excellent evidence of the toxicity and side effects of the antibiotic.

Winn[5] suggested that serologic changes during the treatment of coccidioidomycosis are useful not only for prognosis but as a guide to duration of therapy

The serology of histoplasmosis is not on as firm a foundation. Nevertheless, if a high titer of complement fixing antibodies is present before treatment, we believe that a three- or fourfold decrease in titer is significant when properly determined. The complement fixing titer of the pretreatment serum and sera drawn at monthly intervals during treatment should be compared in simultaneous tests. The first and last sera drawn should be adequate in volume to serve as controls against subsequent sera and should always be run with them. This is a basic principle of serologic testing that is neglected frequently by physicians. There are no serologic tests for patients with cryptococcosis. Serologic methods now available are not helpful in North American blastomycosis or in those patients with histoplasmosis whose initial titers are low.

A pitfall of serial single complement fixation determinations is illustrated by the course of patient 25. Complement fixing antibodies against histoplasmin were present in his serum in a titer of 1:128 before treatment. Serologic study in the same laboratory three months later revealed a complete absence of complement fixing antibodies against both histoplasmin and yeast phase antigen. Blood drawn on the day following this negative report had a complement fixing titer of 1:64 against histoplasmin. If testing had been done on all 3 sera at the same time, such conflicting results would have been unlikely.

The toxicity of amphotericin may not be appreciated fully until many more patients have received it. Our experience indicates that the kidney is affected ost frequently. Urea clearance may be reduced markedly. Azotemia ally disappears when treatment is stopped. Patient 16, however, had n al blood urea nitrogen and urinalysis prior to treatment, but a diminished clearance and elevated blood urea nitrogen more than one year after the ination of therapy. This patient gave no history of previous renal disease his cannot be eliminated definitely. More study of renal toxicity is

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Intravenous amphotericin may produce a hemorrhagic enterocolitis in dogs Three of our patients have had clinical signs and symptoms suggesting a similar toxic effect One patient with persistent epigastric pain following infusion had a hemorrhagic gastroenteritis at autopsy on the day following the last administration of amphotericin Death was due to acute staphylococcal pneumonia and this may have accounted for the gastroenteritis

A mild normochromic anemia developed in many of our patients but this was never significant clinically nor greater than could be accounted for by the underlying disease

Two patients developed profound muscular weakness during the course of intravenous therapy Biopsy of the muscle of one of these patients revealed the presence of myositis Potassium salts given intravenously restored both patients to their previous level of muscular strength The hypokalemia present may have been due to specific toxicity of amphotericin but it could be accounted for by the severe anorexia and vomiting present in both

Some patients who were given a second course of treatment or whose therapy was interrupted for several weeks or more have experienced more severe reactions when treatment was resumed than during the initial course We have

no proof that amphotericin B produced hypersensitivity in any of these patients Until this possibility is better understood, we suggest that re treatment be approached with caution

Side effects from intravenous amphotericin B are many Fever, chills, and nausea are usually controllable by adjunctives Acetylsalicylic acid and antihistaminics are used routinely When side effects are severe or persistent, we believe that 25 mg of sodium succinate ester of hydrocortisone will reduce reactions considerably if injected at the time of starting the infusion or incorporated into the dextrose solution

Local instillation of amphotericin B has been well tolerated Dissolving the antibiotic in 1 per cent procaine made it suitable for local infiltration and injection into suppurated lymph nodes and pyoarthroses No additive was necessary for intrapleural instillation

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THE TREATMENT OF SYSTEMIC FUNGUS INFECTIONS WITH AMPHOTERICIN B*

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Since its introduction four years ago by Gold *et al.*[1] amphotericin B† has received increasing clinical trial in the treatment of virtually all of the systemic mycoses that affect man. Much additional experience will be required before the ultimate value of amphotericin B can be determined, but accumulating clinical data suggest that to date it represents the most effective chemotherapeutic agent currently available for the treatment of these heretofore recalcitrant diseases. This paper deals with our clinical experience to date with amphotericin B in the treatment of systemic mycoses, particularly coccidioidomycosis.

Amphotericin B is isolated from a species of *Streptomyces*.[1] It is a member of a growing family of closely allied polyene antifungal agents, which includes amphotericin A, trichomycin, rimocidin, nystatin, candicidin, ascosin, pimaricin and others. A tentative empirical formula $C_{46}H_{73}NO_{20}$ has been assigned to it.[2] *In vitro* amphotericin B inhibits a wide variety of pathogenic and nonpathogenic fungi in concentrations ranging from 0.5 µg./ml. to 12.5 µg./ml.[1,3] The growth of the majority of the organisms producing systemic mycoses is inhibited by amphotericin B in concentrations as low as 0.02 to 0.5 µg./ml.[1] These include *Blastomyces dermatitidis*, *Blastomyces brasiliensis*, *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis* and the yeast phase of *Sporotrichum schenkii*. Initial toxicity studies indicated that calculated therapeutic levels of the drug could be obtained without serious sequelae in several species of animals.[4] These studies were followed by others designed to evaluate the effectiveness of amphotericin B on experimentally produced fungus infections in animals. Amphotericin B has favorably influenced the course of such infections due to *C. albicans*, *C. neoformans*, *H. capsulatum*, *C. immitis*, *Rhizopus oryzae*, *Aspergillus fumigatus* and some *Volutella* species.[5] Preliminary studies in man indicated that amphotericin B was in general poorly absorbed when given by mouth[5] or intramuscularly. In addition, when the drug was given intramuscularly in therapeutic amounts it proved too irritating for routine use.[12] The currently available preparation of amphotericin B has been most usefully employed intravenously, although in certain instances it has been effective when given by other routes. The drug is highly insoluble in aqueous solutions and the early intravenous preparations consisted of relatively coarse suspensions of the drug.[13] The use of sodium desoxycholate as a solubilizing agent has improved the intravenous preparation considerably; however, true solutions

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† Supplied by Squibb Institute for Medical Research, New Brunswick, N. J.

are still not produced but rather a colloidal dispersion of particles smaller than bacteria[12] The drug is diluted in 500 to 1000 cc of dextrose injection (5 per cent U S P) and administered intravenously over a 6 hour period The initial dose is usually in the range of 5 to 10 mg /day with daily increments of from 5 to 10 mg depending on the patient s tolerance for the drug Once a dosage level of from 0 7 to 1 4 mg /kg per day is reached (in the adult 50 to 100 mg) an attempt is made to sustain it Adjustments of the dosage including complete cessation of the drug for short periods of time administration on alternate days and return to lower dosage levels are employed as necessitated by the patient s reaction Dosages of more than 100 mg of amphotericin B intravenously per day have been tolerated by adults for short periods of time but in our experience toxic manifestations soon necessitate the reduction of this daily dosage

Material and Methods

To date we have treated with amphotericin B 30 patients with various systemic mycoses either under direct observation or in cooperation with other physicians in the community All patients were hospitalized and some or all of the following base line studies were obtained before instituting therapy complete blood count urinalysis liver function studies blood urea nitrogen serum creatinine chest films and other appropriate X rays fungus cultures serologic tests and spinal fluid examinations where applicable These studies were repeated at appropriate intervals during the course of therapy wherever possible Amphotericin B was administered in courses varying from 2 weeks to 5 months in duration The patients have been followed for periods up to 3 years TABLE 1 details our data regarding the patients treated to date TABLE 2 summarizes the results obtained in the treatment of these patients

Coccidioidomycosis

We have treated 18 patients with disseminated coccidioidomycosis Five of these had coccidioidal meningitis Two of these 5 were given amphotericin B intrathecally as well as intravenously One patient succumbed after 7 weeks of therapy All of the other patients have shown various degrees of clinical improvement with clearing of the sensorium disappearance of headaches improvement in the neurological findings and cessation of nausea and vomiting Two returned to gainful employment The spinal fluid findings of these latter patients improved but some laboratory abnormalities have continuously persisted in all including such findings as increased protein pleocytosis elevated pressure and persistent positive complement fixation tests for coccidioidomycosis Two patients have suffered clinical relapse after 7 and 21 months respectively One has been lost to follow up The remaining patient has become asymptomatic but has been followed for too short a period of time to determine his final outcome

The remaining 13 patients had involvement of bone skin lungs or combinations thereof Five of these demonstrated no improvement but of these 5 2 were inadequately treated in the light of our current knowledge 1 patient

receiving the drug intravenously for only 10 days the other solely by the oral route Initially however the remaining patients demonstrated various degrees of improvement some to the point of being considered clinically free of the disease Of this latter group of 8 patients 2 have relapsed 6 and 8 months respectively after discontinuing therapy and in several of the remaining 6 the complement fixation test has remained ominously elevated in spite of their asymptomatic clinical status

Cryptococcal Meningitis

Five patients with cryptococcosis have received amphotericin B orally intravenously and intrathecally Two patients succumbed 1 receiving amphotericin B orally only and the other receiving 1050 mg of amphotericin B intravenously over a 24-day period The remaining 3 patients have shown moderate to marked clinical improvement In 2 of these patients cultures of the spinal fluid are negative 18 months after discontinuation of therapy One of these received amphotericin B intrathecally in a total dosage of 11 3 mg over a 1 month period No abnormalities exist in the spinal fluid of these 2 patients at present and the third has been lost to follow up although her spinal fluid culture was negative at the time of her last visit

North American Blastomycosis

Our experience with North American blastomycosis has been limited to the treatment of one patient with genitourinary and cutaneous involvement This patient was given amphotericin B intravenously over a 22 day period a total dosage of 880 mg This was followed by complete healing of the cutaneous lesions and the disappearance of *B dermatitidis* from the urine No evidence of recurrence was present 18 months after discontinuing therapy

Aspergillosis

Two patients with pulmonary aspergillosis have been treated One a 2½ year old white male with hypergammaglobulinemia improved after receiving 470 mg of amphotericin B intravenously over a 3 month period and 1 remained unchanged after receiving 624 4 mg of amphotericin B over a 15-day period In the latter patient therapy was discontinued because of phlebitis at the site of infusion

Candidiasis

Two patients with candidiasis have been treated One patient with ocular involvement with *Candida parakrusei* following a corneal transplant demonstrated a complete clearing of his infection following topical and intravenous administration of amphotericin B but subsequently lost the eye following failure of the corneal graft to take The other patient had had a chronic generalized infection due to *C albicans* extensively involving the oral mucosa vaginal mucosa and 7 fingernails She had resisted all previous forms of therapy including numerous topical agents and prolonged courses of orally administered nystatin pimaricin trichomycin amphotericin A and a mixture of amphotericin A and B She received 2 courses of amphotericin B intra

TABLE 1
SYSTEMIC MYCOSES TREATED WITH AMPHOTERICIN B

Patient age race sex	Diagnosis	Skin test	Complement fixation	Precipitin	Dosage of amphotericin B	Duration of time
1 T L., 48, WM	Coccidioidal meningitis	Negative 1 100	Serum 1+ 1 16 spinal fluid 1+ 1.8	Negative	10 to 50 mg I V every day 50 mg I V every day	2 weeks 6 weeks
2 W R , 29, WM	Disseminated coccidioidomycosis with cutaneous lesions and meningitis	Negative 1 100	Serum 1+ 1 16, spinal fluid 1+ 1 2	Negative	Avg 25 to 45 mg I V daily Intrathecal avg 0 5 to 0 9 mg q o d	79 days 33 days
3 C S , 26, WM	Coccidioidal meningitis	Negative 1,100	Serum 3+ 1 16	Negative	50 mg I V. every day 0 1 to 1 mg intrathecally twice weekly	10 weeks 7 weeks
4 R B , 26, WM	Coccidioidal meningitis	Negative 1,100	Serum 2+ 1.8	Negative	5 to 50 mg I V every day	5 months
5 S C , 27, Am Indian, M	Coccidioidal meningitis	Negative 1 100	Serum 2+ 1.16, spinal fluid 4+ 1 4	Negative	Suspension 1 5 mg /kg every day	7 weeks

Patient	Total dosage	Results	Toxicity	Remarks
1 T L., 48, WM	2440 mg	Complement fixation tests of spinal fluid after 8 wks treatment 1+ 1 16, afebrile mentally alert, discharged from hospital	Chills, fever, increase in BUN, and drop in hematocrit from 47 to 35	Seven months after treatment relapsed, but lost to follow up
2 W R , 29, WM	1505 mg 7 25 mg	Skin test—Neg , C F serum 1 32, C F CSF—Neg , clearing of sensorium meningismus and headache Return of spinal fluid pressure to normal	Fever, chills, malaise BUN increased from 15 8 to 31 5, intrathecal administration produced transient paresthesias and low back pain	Spinal fluid protein (360) (131) cell and sugar—(36) have remained abnormal Still being treated
3 C S , 26 WM	3500 mg 9 5 mg	Some improvement in laboratory findings, spinal fluid protein, cells and pressure decreased, over all condition unchanged	Vomiting following I V administration (vomiting also part of clinical picture prior to treatment)	Transferred to USVA Hospital in Houston, Texas, further contact lost

4 R B 26 WM	6200 mg	Clinically well skin test 1+ 1 10	Malaise tremor and back pain	Returned to gainful employment Then 1 year 9 months later developed vertigo and ataxia Cell count in spinal fluid—134 WBC protein 210 mg % given amphotericin B 0 2–0 8 intrathecally q o d Developed pelvic girdle pain and (?) arachnoiditis drug was therefore discontinued C F 2 years after treatment serum 1 8 CSF 1 4 Continues to have ataxia and falling to right
5 S C Am Indian M	4100 mg	Expired		

Patient	Drug levels
1 T I 48 WM 2 W R 29 WM 3 C S 26 WM	Spinal fluid 0 20 to 0 56 μg /ml
4 R B 26 WM 5 S C 27 Am Indian M	Serum 1 55 to 2 65 μg /ml spinal fluid 0 to 0 74 μg /ml

Patient age race sex	Diagnosis	Skin test	Complement fixation	Precipitin
6 D C 44 WM	Possible coccidioidal meningitis	2+ 1 100	Serum 1+ 1 16 spinal fluid negative	Negative
7 A L 12 Mexican F	Coccidioidal granuloma of radial head and left midtarsal area	—	Serum 1+ 1 256	Negative
8 G F 6 Mexican F	Possible coccidioidal granuloma left ankle	Positive 1 100	Serum 2+ 1 4	Negative
9 D B 5 WM	Disseminated coccidioidomycosis	Negative	Positive 1 128	Negative
10 L T 53 WM	Disseminated coccidioidomycosis with peripheral granuloma	Positive 1 100	1 128	Negative
11 R D 33 WM	Coccidioidomycosis with granuloma of neck	4+ positive 1 100	Serum 4+ 1 32	4+ 1 10
12 J M 24 Mexican M	Disseminated coccidioidomycosis	Positive 1 100	Serum 1 1024	Negative
13 S D 36 WM	Disseminated coccidioidomycosis with destruction of several carpal bones	Positive 1 1000	Serum 3+ 1 128	Negative

TABLE 1—*Continued*

Patient	Dosage of amphotericin B	Duration of time	Total dosage	Results
6 D C, 44 WM	50 mg I V every day	2 months	3000 mg	Remission of symptoms of headache and vomiting
7 A L 12 Mexican F	50 mg I V every day	3 weeks	1050 mg	Healing of sinus tract of foot improvement in sinus tract of arm after radial head resection
8 G F 6, Mexican F	15 mg I V (1 mg /kg) every day	10 days	150 mg	No improvement
9 D B, 5 NM	15 mg q o d I V 20 mg q o d I V	2½ months 1¼ months	500 mg	Partial clearing of chest X ray improved sense of well being
10 L T 53, NM	5 to 40 mg I V daily 20 mg I V on average 3X/week	9 days 4 months	1025 mg	Decreased size of peripheral granulomata C F unchanged
11 R D, 33 NM	50 mg I V every day or every other day	2 months	2250 mg	Clearing of lung fields cessation of cough, afebrile, increased weight and strength no adenopathy, granuloma of neck healed
12 J M, 24, Mexican M	5 to 40 mg I V daily 10 to 30 mg on average q o d	8 days 36 days	510 mg	Much improved feeling of strength and well being C F decreased to 1 8 Weight gain of 2 5 kg
13 S D, 36, NM	25 to 140 mg I V every day	4 months	5450 mg	Complement fixation dropped to 3+ 1 32, little clinical change

Patient	Toxicity	Remarks	Drug levels
6 D C, 44 WM	Increase of BUN nausea	Patient had had bilateral upper lobectomies for pulmonary coccidioidomycosis and subsequently developed headache and morning vomiting with elevated spinal fluid pressure, increased protein, and cells cultures complement fixation, and precipitin tests of spinal fluid all negative, patient has been asymptomatic for approximately 10 months since discharge from hospital	—
7 A L 12 Mexican F	Nausea vomiting fever		—
8 G F 6 Mexican F	Chills fever, nausea	Diagnosis of coccidioidomycosis not confirmed by culture	—
9 D B, 5 NM	Chills fever, nausea		—
10 L T 53 NM	BUN increased from 14 to 33	Hb rose from 11 6 to 15 4	—

11 R D 33 NM	Nausea vomiting fever BUN increased from 6 to 26	Complement fixation rose to serum 2+ 1 256 6 months after cessation of treatment developed recurrence of granuloma of neck	—
12 J M 24 NM	Nausea vomiting fever BUN increased from 15 5 to 34	Returned to gainful employment	—
13 S D 36 NM	Occasional low grade fever	Hematocrit fell from 51 to 45 BUN rose from 19 to 44 later dropped to 10 while still on drug recently treated with intra arterial perfusion of amphotericin B Results pending	Serum 1 2 mcg /ml

Patient age race sex	Diagnosis	Skin test	Complement fixation	Precipitin
14 J H 46 WM	Disseminated coccidioidomycosis with skin lesions	Negative 1 100	Serum 2+ 1 32	4+ 1 40
15 C B 55 WM	Disseminated coccidioidomycosis with coccidiodal lymphadenitis	Negative 1 100	Serum 1+ 1 4	4+ undiluted
16 B A 23 WM	Disseminated coccidioidomycosis and pleurocutaneous fistula	Negative 1 100	Serum 2+ 1 256	Negative
17 F J C 40 WM	Disseminated coccidioidomycosis	Positive 1 100	Serum 2+ 1 32	—
18 A S 26 NM	Disseminated coccidioidomycosis with bone and skin involvement	Negative	Serum 2/22 1 2048 5/1 1 16000 6/6 1 4096 7/10 1 8192	—
19 R R 2½ WM	Pulmonary aspergillosis with mass in neck and draining thoracic sinus			
20 R T 57 WM	Pulmonary aspergillosis			
21 F K 29 WF	Candidiasis oral—2 years nails and vagina—6 months	—	—	—
22 R E 48 WM	*Candida parakrusei* infection of eye after corneal transplant			
23 T H 29 WF	Cryptococcal meningitis			

Patient	Dosage of amphotericin B	Duration of time	Total dosage	Results
14 J H 46 WM	50 mg I V every day	3 months	4550 mg	C F dropped to 2+ 1 16 precipitin dropped to 0 afebrile no clinical evidence of recurrence complete healing of skin lesions lung infiltrate unchanged now working
15 C B 55 WM	50 mg I V every day	25 days	1250 mg	C F 2+ 1 16 regression of lung lesions clinically well and returned to work
16 B A 23 WM	5 to 15 gm /OS every day	3½ months	1050 gm	No change

TABLE 1—*Continued*

Patient	Dosage of amphotericin B	Duration of time	Total dosage	Results
17 E J C, 40, WM	5 to 50 mg I V every day 45 to 60 mg I V every day	10 days 26 days	1650 mg	Marked reduction of swelling dorsum right hand and scrotal nodules, symptomatically improved complement fixation unchanged, returned to work
18 A S, 26 NM	5 to 50 mg I V every day 50 mg I V every day	8 days 3½ months	5350 mg	Gained 25 lb (131 to 156), increased appetite, strength, elevated hemoglobin from 8 to 13.6 gm Healing of abscesses low back and closure sinus tract from low back to left femoral head area
19 R R, 2½ WM	1 to 10 mg /kg I V every day 1 gm /OS every day	3 months 3 months	470 mg 93 gm	Partial healing of draining sinus of chest partial clearing of lung infiltrate *general clinical improvement*
20 R T, 57 WM	8.4 to 40 mg I V every day 50 mg I V every day	9 days 6 days	324.4 mg 300 mg	Therapy discontinued because of phlebitis no improvement at that time
21 E K, 29, WF	5 to 50 mg I V daily 20 mg I V q o d 5 to 50 mg I V daily 25 mg I V q o d	5 days 16 days 5 days 8 days	265 mg *190* mg	Vaginal and mucous membrane lesions cleared Nails greatly improved Striking clinical improvement
22 R E., 48, WM	5 to 10% solution topically 30 to 80 mg I V every day	26 days 7 days	410 mg	*Candida* infection cleared
23 T H 29, WF	0.25 mg /kg I V every day 0.50 mg /kg I V every day 0.75 mg /kg I V every day 1.00 mg /kg I V every day	5 days 5 days 3 days 8 days	840 mg	Spinal fluid cultures became negative March 26, drug started April 13, *returned home, clinically fairly well*

Patient	Toxicity	Remarks	Drug levels
14 J H, 46, WM	Nausea for first 10 doses, none thereafter, mild persistence of backache, fever, and vomiting for first 13 doses, none thereafter	No rise in BUN prophylactic aspirin and antihistamines did not prevent fever with drug administration	—
15 C B, 55, WM	Nausea, vomiting and fever	BUN rose from 15 to 28 One year later still well, working and active	
16 B A, 23, WM	None observed	Refused IV medication	Serum 3.65 mcg /ml, urine 5.0 mcg /ml Serum 0.0 mcg /ml
17 E J C, 40, WM	Hemoglobin dropped from 14 to 11.1 gm	Has remained clinically well to date and returned to work Recurrence of lesion dorsum right hand C F dropped to 1 8	—

18 A S 26 NM	Chills fever nausea decreased with progressive administration rise in BUN to 35 mg %	Excellent clinical improvement despite poor serologic response Clinically well working last C F 10/58 1 1024	—
19 R R 2½ WM	Vomiting	Patient had hypergammaglobulinemia and history of many previous infections	Serum 0 53 to 1 82 μg /ml, spinal fluid 0 00 μg /ml urine 1 05 to 1 41 μg /ml
20 R T 57 WM	Hemoglobin dropped from 15 5 to 13 2 gm chills fever	Current status unknown	
21 E K 29 WF	Chills and fever malaise	5 months after therapy developed recurrent paronychia and was re treated	
22 R I 48 WM	None	Grafts sloughed and eye lost	
23 T H 29 WF	Vomiting fever rise in BUN	While on drug had 4 grand mal seizures and became semicomatose now home and clinically well Lost to follow up	Serum 0 to 2 2 μg /ml, spinal fluid 0

Patient age race sex	Diagnosis
24 M S 30 WF	Cryptococcal meningitis
25 L R 58 WM	Cryptococcal meningitis
26 W M 42 WM	Cryptococcal meningitis
27 J P 23 NM	Cryptococcal meningitis
28 T M 50 WM	Mycetoma (Madura foot) due to *Monosporium apiospermum*
29 W S 62 WM	North American blastomycosis
30 J Q 58 Mexican F	Sporotrichosis

Patient	Dosage of amphotericin B	Duration of time	Total dosage	Results
24 M S 30 WF	10 to 16 gm /OS every day	16 days	224 gm	Expired
25 L R 58 WM	15 to 50 mg I V every day	9 days	1050 mg	Expired
	50 mg I V every day	15 days		
26 W M 42 WM	5 to 50 mg I V every day	7 days	2300 mg	Clinically well with disappearance of headache and clearing of sensorium
	50 mg I V every day	39 days		
27 J P 23 NM	5 to 40 mg I V every day	5 days	3150 mg	Dramatic clinical improvement, spinal fluid cultures negative at one month post treatment
	50 mg I V every day	60 days		
	0 1 to 1 mg intrathecally every other day	1 mo		
28 T M 50 WM	5 to 50 mg I V every day	10 days	1050 mg	Decreased drainage from nodules on foot, otherwise no change
	50 mg I V every day	13 days		

TABLE 1—*Continued*

Patient	Dosage of amphotericin B	Duration of time	Total dosage	Results
29 W S, 62, WM	12 5 to 40 mg I V every day 50 to 75 mg I V every day	4 days 18 days	1270 mg	Cutaneous lesions healed epididymitis cleared, urine culture for *B dermatitidis* negative 2 weeks post treatment
30 J Q, 58, Mexican F	600 to 3000 mg /OS every day 5 mg /day I V	57 days 2 days	147 6 gm 10 mg	Complete clinical cure

Patient	Toxicity	Remarks
24 M S, 30, WF	None	
25 L R, 58, WM	Chills, fever, nausea, vomiting	Underlying pathology of multiple myeloma preceded onset of cryptococcal meningitis, course complicated by a *Proteus mirabilis* septicemia
26 W. M, 42, WM	BUN rose from 16 to 28 mg per cent, chills, fever from 100 to 102° F, hemoglobin dropped from 18 to 10 gm	*Before*: Spinal fluid cells, 1,100, protein 1+, Pandy, sugar, low; pressure, 400 18 months after discontinuing therapy, patient is clinically well and spinal fluid has been completely normal for 4 months.
27 J P, 23, NM	Nausea, chills, vomiting, fever	18 months after discontinuing treatment patient remains well clinically and by laboratory tests with regard to his meningitis.
28 T M, 50, WM	Nausea vomiting, chills, fever, BUN rose to 42 8 mg per cent	No significant improvement, given intra arterial amphotericin B, awaiting results
29 W S, 62, WM	Chills nausea, fever, vomiting, hemoglobin dropped from 15 5 to 13, creatine rose from 1 2 to 2 3	Therapy stopped because of localized phlebitis, 1 yr later—remains clinically well, skin and urine cultures negative
30 J Q, 58, Mexican F	Intravenous amphotericin B produced fever, chills headache, nausea, malaise	Follow up of patient 13 months later shows patient clinically well

venously 265 mg over a 21-day period and, 5 months later, 190 mg over a 13-day period During the first course of treatment all infected nails were avulsed The oral and vaginal lesions improved rapidly, and all nails appeared to be returning normally However, 5 nails subsequently developed evidence of infection and, 5 months later, these infected nails were again avulsed and the patient received a second course of amphotericin B Again, all nails appeared to be returning normally, however, 2 nails have recently become infected

Mycetoma

A single patient with far advanced mycetoma (Madura foot) due to *Monosporium apiospermum* was treated over a 20 day period with intravenous amphotericin B, with essentially no improvement

TABLE 2
SUMMARY OF RESULTS OF PATIENTS TREATED WITH AMPHOTERICIN B

	Total	Improved	Improved then relapsed	Unchanged
Disseminated coccidioidomycosis	18	7	4	7
Meningitis	5	1	2	2
Bone skin lung	13	6	2	5
Cryptococcal meningitis	5	3	—	2*
Pulmonary aspergillosis	2	1	—	1
Candidiasis	2	2	—	—
Mycetoma due to *Monosporium apiospermum*	1	—	—	1
North American blastomycosis	1	1		1
Sporotrichosis	1	1*	—	—
Total	30	15	4	11

* One patient treated with oral amphotericin B only

Sporotrichosis

One patient with the localized lymphangitic type of sporotrichosis involving the hand, forearm, and arm with multiple ulcerating lesions was treated with oral amphotericin A and B in essentially equal parts of each to a total dose of 147 6 gm over a 57 day period This was followed by complete resolution of all lesions Thirteen months after discontinuation of therapy the patient remains clinically well

Discussion

It should be stressed at the outset that the evaluation of a fungicide on the course of the systemic mycoses in man is extremely difficult because of many factors the small number of patients available, the reluctance on the part of the clinician to withhold a potentially effective medication to form ideally matched control groups, the difficulty in obtaining unbiased observations, the lack of knowledge concerning many facets of the natural course of the systemic mycoses and, finally, the inevitable loss of patients from prolonged follow up

since adequate follow-up periods must be measured in years before preliminary opinions can be substantiated. Realizing these shortcomings, we nevertheless feel that there is still considerable value in presenting our clinical experience to date, even though many of our conclusions must be of a tentative nature.

Our clinical experience with amphotericin B has been primarily with the treatment of disseminated coccidioidomycosis.[12] Amphotericin B, when given intravenously, appears definitely to influence favorably the course of this disease in some cases, so that a patient with progressive disseminated coccidioidomycosis, shortly after the inception of treatment, may develop an improved feeling of well being, increased appetite, weight gain, defervescence, and healing of cutaneous, osseous, and pulmonary lesions, and eventually appear clinically well. This has occurred particularly following the initial treatment of that group of patients having disseminated disease with skin and soft tissue or lung involvement, or both. Of the 13 patients treated to date, 6 have improved and remained symptom free for periods as long as 3 years. Two of the 5 who did not improve received grossly inadequate treatment according to our present concepts. The remaining 5 patients either did not respond or have relapsed following cessation of therapy. Again it should be stressed that those patients who are at present clinically well have not been followed long enough for final evaluation, moreover, we have no comparable control group upon which to interpret these seemingly favorable results.

Although coccidioidal meningitis thus far has been consistently fatal with patients generally succumbing within a period of 2 years, some of the more chronic forms have persisted for 10 years and longer. This has tended to make the evaluation of any form of therapy extremely difficult in this particular complication of coccidioidomycosis.

The ultimate value of amphotericin B in preventing meningeal involvement, which is the major cause of death in Caucasians with disseminated disease and which, in some series of disseminated coccidioidomycosis, occurs with a frequency of 25 per cent, is unknown at this time. Evidence to date suggests that amphotericin B can produce remissions and perhaps prolong life in the patient with coccidioidal meningitis. The ultimate rate of actual cure of patients with coccidioidal meningitis is, however, undetermined at this time.

Improvement occurred in 3 of our 5 patients with coccidioidal meningitis, in 2 instances to the point where the patient was relatively symptom free and was able to return to gainful employment. However, abnormalities persisted in the spinal fluid in all of these instances. The significance of this has become more ominous since 2 of these 3 have already developed clinical relapse. These results are not as favorable as those reported recently by Winn,[14] who has detailed the treatment of 6 patients with meningitis due to *C. immitis*. Excellent results were obtained in 3 of the 6 patients, these patients becoming clinically asymptomatic and their spinal fluid abnormalities returning completely to normal. Some of these have been followed for periods as long as 18 months. Winn stresses the simultaneous intrathecal and intravenous administration of amphotericin B. The value of the intrathecal use of amphotericin B is still open to question, even though the drug is known to be poorly transported across the blood brain barrier into the spinal fluid.[14,15,16] Apparently quanti-

ties reach the infected portions of the meninges at a level sufficient to produce clinical remissions in some instances of meningitis due to C *neoformans* C *immitis* and B *dermatitidis* [11,21]. Because of the serious nature of coccidioidal meningitis and until more definite data have been accumulated concerning the value of the intrathecal use of amphotericin B, it seems desirable concurrently to treat such patients both intravenously and intrathecally. The drug is at present recommended for intrathecal administration in dosages not to exceed 1 mg every other day in the adult. This dosage is attained by starting with 0.1 mg and slowly increasing to the maximum depending upon the patient's reactions. It is dissolved in 2 to 3 cc of distilled water or spinal fluid. A total dose of 44.42 mg has been given in 2 courses over a period of 234 days [14]; however the development of arachnoiditis in this patient interfered with its regular use.

The exact optimal dosage for the treatment of coccidioidomycosis is still undetermined and the length of treatment still depends on evaluation of the clinical status of the patient. At present however it appears to be in the range of 2000 to 4000 mg although some patients have responded to lower doses and in some instances have required up to 10 000 mg total dosage. The ideal goal that should be sought is complete disappearance of all clinical evidence of disease with the regaining of the patient's sense of well-being and former weight. Furthermore reversion of the laboratory findings to normal including sedimentation rate hemoglobin complement fixation test for coccidioidomycosis and skin test for coccidioidomycosis is highly desirable but in our experience it has not been generally attainable particularly in respect to complement fixation tests for coccidioidomycosis. Laboratory evidence of kidney damage may necessitate the temporary cessation of amphotericin B therapy but to date it has not posed a serious problem in the over all use of the drug except insofar as it limits the total amount of amphotericin B that can be administered in a specific period of time. Sclerosis of the superficial veins has occurred in some instances to such a degree as to be a serious handicap to the continued administration of amphotericin B.

In the untreated patient with coccidiodomycosis the properly performed skin test and complement fixation tests at intervals of about one month have permitted one to make a more accurate prognosis than by relying on the clinical observations [2]. For example a highly positive skin test and a negative complement fixation test indicate an excellent prognosis and a negative reaction to a skin test and a high titer response to the complement fixation test suggest death by dissemination.

These tests in patients receiving treatment with amphotericin B have not reflected the patient's changing status at a sufficient rate of speed to act as guides to therapy. The skin test to coccidioidin may remain negative throughout the course of therapy or may revert to low positivity in both instances the patients may appear clinically cured. The complement fixation test for coccidioidomycosis may decrease greatly over several months decrease moderately in titer may then return to the previous status may remain essentially the same or may increase temporarily while clinically the patient during this same period may become asymptomatic may show no improvement or may

become clinically asymptomatic but later develop local recurrence of the disease

Which trend in the laboratory and skin tests and their combinations may indicate *biological or clinical cure or* on the other hand impending relapse and thus indicate the need for retreatment before clinical manifestations become apparent are questions that still await clarification At present the criteria for re treatment are based primarily on the development of clinical symptomatology Evidence in the literature suggests that *C immitis* can develop a significant degree of resistance to amphotericin B *in vitro*[23] This possibility however has not been encountered at a clinical level to date

Finally it should be stressed that amphotericin B supplements but does not supplant those measures that are referred to as supportive These include bed rest a high protein high vitamin high caloric diet excellent nursing care and drainage of the subcutaneous abscesses and other surgical procedures where indicated Many instances of cures attributed to promising fungicidal agents may be attributed in retrospect to the intensified application of these measures by the interested investigator

Amphotericin B appears to be the most effective chemotherapeutic agent available for the treatment of coccidioidomycosis Its exact status in this respect however is still to be determined It appears capable in some instances of arresting the progress of disseminated disease eliminating all clinical symptoms and even reversing laboratory evidence of dissemination The exact rate of relapse is unknown but probably will increase as longer periods of observation are attained The drug appears to produce remissions in patients with coccidioidal meningitis but here again the percentage of patients who ultimately will be cured appears extremely small

Amphotericin B may prove to be more effective in the prevention of dissemination than in its treatment but this point needs documentation Until more definite knowledge is available it seems desirable to treat a variety of clinical situations in which dissemination is a likely possibility and is known to occur with a high frequency This includes patients with severe primary coccidioidomycosis that does not show evidence of improvement within a few weeks of conservative management or who demonstrate a weak or negative skin test coupled with a rising complement fixation titer the primary pulmonary complex in the Negro Filipino and Mexican primary coccidioidomycosis in the pregnant woman the diabetic the patient with a lymphoma a patient who is receiving corticosteroids or in other clinical conditions where the host defense mechanisms are basically impaired and finally as coverage for surgical procedures involving diseased tissues[14, 24]

Our experience with the other systemic mycoses has been more limited The one patient with North American blastomycosis promptly and rapidly improved following the administration of a comparatively small quantity of amphotericin B this provides additional support to the tentative conclusion that amphotericin B appears to be of considerable value for the treatment of this disease entity[2, 25, 26]

No spinal fluid abnormalities have persisted in the three patients with cryptococcal meningitis who achieved clinical cure thus their *prognosis appears*

favorable, but further follow up will be necessary to determine their ultimate outcome. These findings are similar to those of many other investigators.[16-20] Assessment of the ultimate curative value of amphotericin B for cryptococcal meningitis must, however, await longer follow up periods.

Two patients with pulmonary aspergillosis have been treated with amphotericin B. One, a 2½ year-old Caucasian boy, showed partial resolution of his pulmonary lesions and disappearance of the organisms from exudates of a draining thoracic sinus. The other patient was unable to complete an adequate course of therapy because of severe phlebitis at the sites of infusion. No conclusions can be drawn from this limited experience except that the drug is worthy of further clinical evaluation in this disease.

Amphotericin B appears to offer promise for the treatment of several recalcitrant forms of candidiasis. The one patient who had extensive infection due to *C. albicans* involving the oral and vaginal mucous membranes and severe deformities of seven fingernails who had resisted all forms of topical and oral therapy, promptly responded to amphotericin B given intravenously. A recurrence involving two nails however, developed following two courses of therapy. The second patient achieved rapid sterilization of an ocular infection due to *C. parakrusei*. The rapid response of a variety of candidal infections to relatively small doses of amphotericin B suggests that this drug has great potential value in the management of many heretofore chronic, recalcitrant, and occasionally fatal candidal infections.[27-29]

One patient with primary cutaneous sporotrichosis with numerous ulcerative granulomatous lesions extending up to the forearm and upper arm responded to amphotericin B administered orally. Although potassium iodide remains the drug of choice for the treatment of this disease amphotericin B may be of considerable value in the treatment of such cases when the organism does not respond to adequate doses of iodine or the patient has become intolerant to the administration of iodine.

We have had no experience in the treatment of histoplasmosis or South American blastomycosis or chromoblastomycosis. The current literature indicates that the drug is of extreme value for the treatment of several types of histoplasmosis[20,30-37] and South American blastomycosis[38-41] particularly in those instances where the organism has become resistant to sulfonamide therapy. Chromoblastomycosis has been cured by local injections[42,43] and by tattooing[44] the material into the lesion.

Route of Administration

The intravenous route presently appears to be the most satisfactory method of administering amphotericin B. It must be stressed that the initial dose should be in the range of 5 to 10 mg per day. The dosage may then be increased by increments of 5 to 10 mg depending upon the reactions of the patient. With the initial infusion some patients will develop fever shaking chills anorexia nausea vomiting chest pain and anxiety, with or without headaches. If these symptoms become severe the administration should be discontinued until the following day and again started at essentially the same dosage as otherwise patients may progress to seizures and shocklike states

In general patients tend to develop a tolerance to subsequent administrations including the addition of small increments of the drug

It is preferable to administer the drug over a 4 to 6 hour period Although the drug may be given over a shorter period of time this procedure is not without risk The inadvertent administration of 20 mg of the drug over a 60-min period was followed by grand mal convulsions ventricular fibrillation and cardiac arrest that fortunately responded to a sharp blow on the chest [44]

Chronic cumulative toxicity of the drug developing after prolonged administration is manifested by the development of increasing malaise nausea vomiting and anorexia for increasing lengths of time following each administration of amphotericin B When these symptoms interfere with the nutrition of the patient to such a degree that it becomes apparent that he is not consuming an adequate daily diet the drug should be discontinued temporarily and reinstituted at a lower level either daily or on alternate days depending on the patient's subsequent response

At essentially this same time or even shortly before the clinical evidence of chronic toxicity occurs laboratory studies have demonstrated kidney damage as shown by consistent elevation of the blood urea nitrogen and creatinine levels

Until a better understanding of the nature of the kidney damage produced by the administration of amphotericin B is forthcoming it has been our policy to interrupt therapy until the blood urea nitrogen and creatinine have returned to normal These laboratory abnormalities in our experience to date invariably have returned to their pretreatment levels following cessation of therapy This however has not always been the case (W A Winn personal communication) The need for clarification of the damage if any to the kidney of either a temporary or permanent nature becomes of additional importance as accumulated experience suggests that several of the deep mycoses may require repeated courses of amphotericin B to maintain the patient symptom free A battery of kidney function tests was performed in two of our patients in an effort to elucidate the nature of the kidney lesions These indicated an associated decreased rate of glomerular filtration and a decreased renal plasma flow

An annoying complication and occasionally a limiting factor in the continued administration of amphotericin B is the development of phlebitis at the site of infusion It is therefore important if a prolonged course is contemplated to utilize the most distal vein first using a small needle and a slow rate of infusion We have had no experience with the addition of heparin to the infusion in an effort to prevent phlebitis [14, 4] Other manifestations of toxicity reported in the literature include transitory vestibular disturbances flushing sweating fatigue drowsiness [45] hypokalemia in one patient [44] generalized pain weight loss a drop of the platelet count to 20 000 and anaphylactoid shock [4] In three of our patients we have noted a persistent drop in the hematocrit ranging from 6 to 10 U It is recommended that appropriate hematological and renal function studies be done periodically on all patients receiving prolonged therapy with amphotericin B

Conclusions

Thirty patients with a variety of systemic mycoses have been treated, including coccidioidomycosis, cryptococcosis, North American blastomycosis, candidiasis, aspergillosis sporotrichosis, and mycetoma From our experience, as well as from that reported in the literature, amphotericin B appears to be of significant value in the treatment of coccidioidomycosis, cryptococcosis, North and South American blastomycosis histoplasmosis sporotrichosis candidiasis and chromoblastomycosis Its exact therapeutic status awaits further detailed clinical experience

The drug is most effectively given intravenously in dosages of 0 7 to 1 4 mg / kg daily or on alternate days The high incidence of acute and chronic toxicity associated with the administration of the drug makes close medical supervision mandatory

In general total effective dosage depends on the nature of the infecting agent and the clinical status of the patient The optimum total dosage appears to be in the range of 2000 to 4000 mg for the treatment of coccidioidomycosis in the adult

The most common manifestations of toxicity have included anorexia nausea vomiting anxiety chest pain rising blood urea nitrogen and a fall in hematocrit Generally these symptoms have disappeared upon temporary cessation of therapy

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Discussion of the Paper

Comment May I add a brief summary of my own experience with the treatment of the deep mycoses with amphotericin B I have had the opportunity to treat successfully two cases of South American blastomycosis and one case of Madura foot produced by Madurella with bone involvement

One case of South American blastomycosis had secondary cutaneous lesions produced by hematogenous dissemination of the parasite and the other case presented pulmonary infection with secondary manifestation in the left eye left epididymis and the central nervous system Both cases had been unsuccessfully treated previously with sulfonamides drugs that usually clear up the lesions of South American blastomycosis

The patient with Madura foot had had this condition for more than fifteen years and had been treated with different types of chemotherapy He was subjected simultaneously to a conservative surgical treatment removing the bone fragments and to amphotericin B injected intravenously

These 3 patients healed within a period of 2 to 3 months and did not show any relapses in the follow up of 1 1/2 years All of them presented some degree of side effects but only one presented toxic manifestation which obliged us to interrupt the treatment for 3 day

One more case of South American blastomycosis was treated successfully in Argentina and four cases were treated with the same results in São Paulo Brazil

Two cases of chromoblastomycosis healed by means of amphotericin B injected locally have been reported from Bellevue Hospital New York N Y I suggest the same type of treatment for rhinosporidiosis which is also a localized deep mycosis

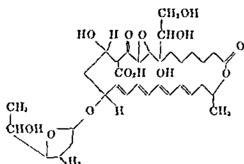
THE TREATMENT OF SYSTEMIC MYCOSES WITH ORALLY ADMINISTERED PIMARICIN: PRELIMINARY REPORT*

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Pimaricin is a new crystalline antifungal antibiotic derived from *Streptomyces natalensis*, originally isolated from a soil sample obtained near Pietermaritzburg, Natal, Union of South Africa. Its chemical and physical properties have been characterized by Struyk *et al.*[1] and by Dekker and Ark.[2] It is a tetraene antibiotic and is related to, but not identical with, rimocidin, antimycoin, nystatin, and chromin. These tetraenes are members of a larger group of antibiotics, the majority of which possess fungicidal activity, and include such compounds as amphotericin, trichomycin, ascosin, and candicidin.

An empirical formula of $C_{30-3}H_{45-50}NO_{13}$ was assigned early to pimaricin.[1] More recently, however, Patrick *et al.*[3] have elucidated the structural formula of pimaricin[3,4] (see formula below), the first of the many polyene antifungal antibiotics to have its structure so determined.



It is insoluble in water, but suspensions may be sterilized for 20 min. at 110° C. with only a moderate reduction in potency. The ultraviolet absorption spectra of pimaricin show considerable similarity to the absorption spectra of both nystatin and amphotericin, but pimaricin can be distinguished readily from these antibiotics by paper chromatography.

The effect of pimaricin is primarily fungicidal, and it possesses no significant bactericidal activity. *In vitro*, it inhibits a wide variety of pathogenic and nonpathogenic fungi in concentrations of from 1 to 10 $\mu g/ml$.[1] The minimum concentrations of pimaricin found in our laboratory to inhibit *in vitro* growth of some of these fungi are summarized in TABLE 1.

Experimentally induced resistance to amphotericin B has been produced in *Candida albicans* and *C. immitis*, but these same organisms showed no in

* This study was supported in part by a grant from the New Drug Institute, New York, N. Y., which also supplied the pimaricin as 200 mg. enteric coated tablets of the sodium salt of pimaricin.

creased cross resistance to pimaricin.[4] Oral toxicity studies in rats demonstrated that 50 to 70 mg. of pimaricin per kilogram could be administered daily for periods of 5 to 10 weeks with no adverse effects; however, doses of 500 mg./kg. produced diarrhea, serious growth inhibition, and a 30 per cent mortality within a 2 week period.

The acute intraperitoneal toxicity of pimaricin was found to be only one tenth that of nystatin, but its oral toxicity was found to be two to three times greater in rats, mice, and guinea pigs. It was hypothesized that perhaps pimaricin was better absorbed from the gastrointestinal tract.[4]

To date amphotericin B has proved to be the most effective fungicidal agent available for the treatment of coccidioidomycosis, North American blastomycosis, cryptococcosis, and histoplasmosis. The fact that the intravenous route of administration has been the only satisfactory means of administering the

Table 1

Inhibition of Various Fungi in Vitro by Pimaricin

Fungus	Minimum inhibitory concentration (µg./ml.)
Microsporum canis	2.5–5
Microsporum audouini	2.5–5
Candida albicans	2.5
Cryptococcus neoformans	3
Trichophyton mentagrophytes	10
Coccidioides immitis	2.5–5
Blastomyces dermatitidis	1
Histoplasma capsulatum	1
Sporotrichum schenckii	2.5
Nocardia asteroides	more than 1,000
Mucor sp. (soil isolate)	1.1–10
Aspergillus fumigatus	1–10

drug except in specialized situations, together with the high incidence of acute and chronic toxic reactions, presents serious drawbacks to its use. Accordingly an antifungal antibiotic effective by the oral or intramuscular route would be of considerable advantage in the treatment of the systemic mycoses. The response of a patient with disseminated coccidioidomycosis in preliminary evaluation of orally administered pimaricin (case 2, Table 2) to the use, yeast flora of the G.I. tract also suggested that enough of its effect on the be absorbed via this route to be useful in the treatment of the systemic mycoses. This led to a consideration of the possibility of utilizing pimaricin as a potential oral agent for the treatment of systemic fungus infections in man.

This paper presents our findings in ten patients with various mycoses who received pimaricin orally.

Materials and Methods

The drug was administered orally in the form of enteric-coated tablets of the sodium salt of pimaricin. The dosage varied in the adults from 200 to 1000 mg. daily, depending upon the patient's tolerance to the drug. Among the patients treated to date were 7 with disseminated coccidioidomycosis, 2 with

TABLE 2
SUMMARY OF RESULTS OF TREATMENT OF VARIOUS MYCOSES WITH PIMARICIN

Patient, age race sex	Disease	Dosage (mg /day)	Duration (days)	Total dose	Result	Toxicity	Comments
1 J M 28 Mexican M	Disseminated coccidioidomycosis	200	24	4800 mg	No change	Anorexia nausea vomiting	Blood urea nitrogen hematocrit complement fixation test for coccidioidomycosis unchanged
2 R D 34 NM	Disseminated coccidioidomycosis	200–1000	154	111 2 mg	Complete healing of granuloma of neck 2 months after instituting therapy with pimaricin	Mild diarrhea, nausea vomiting	Complement fixation test for coccidioidomycosis dropped from 1 2048 to 1 64 Five months after discontinuation of therapy the patient relapsed
		400	180	72 gm	Healing of granuloma of neck, partial regression of regional node	Could not exceed 400 mg daily without severe diarrhea	Despite healing of granuloma regional lymph node remains enlarged and soft
3 A S, 28, NM	Disseminated coccidioidomycosis	50–250	13	2 6 gm	No change	Nausea, vomiting severe anorexia	Drug discontinued because of toxicity
4 B A 26 WM	Disseminated coccidioidomycosis	50–600	64	28 3 gm	No change	Anorexia nausea diarrhea and at lower dosage levels flatulence	Drug discontinued because of toxicity
5 S D 36 NM	Disseminated coccidioidomycosis	600–1000	50	46 gm	Showed initial apparent healing by X ray of osteomyelitis of wrist but subsequently relapsed despite continued therapy	Occasional loose stools	Patient received intramuscularly 0 25 mg of pimaricin in 0 5 cc of saline This produced marked induration and pain
6 R B 35 WM	Coccidioidal meningitis	300–1000	130	96 6 gm	No change	Nausea and diarrhea at doses greater than 800 mg daily	None
7 G S, 67, WM	Disseminated coccidioidomycosis	300–1000	104	89 4 gm	No change	Mild anorexia and loose stools	During the therapy the hemoglobin dropped from 15 7 to 13 1 gm

8 T M 52 WM	Mycetoma due to *Monosporium apiospermum*	200–800	61	28 2 gm	No change	Nausea and vomiting at doses greater than 600 mg daily, flatulence and mild anorexia at doses less than 600 mg / day	None
9 D C 3½ WF	Disseminated muco-cutaneous candidiasis	25–75	70	36 75 gm	Early apparent improvement with partial regression of muco cutaneous lesions and apparent replacement of 2 infected nails by new normal nails	None	Too early to determine effect of drug
10 T K 29, WF	Disseminated muco-cutaneous candidiasis	400	20	8 gm	No change	Severe diarrhea	Drug discontinued because of toxicity

disseminated mucocutaneous candidiasis and 1 with a mycetoma due to *Monosporium apiospermum* The maximum daily dose was determined by the development of clinical evidence of intolerance Courses have varied in duration from 13 days to 334 days

Appropriate laboratory studies including fungal cultures complete blood counts urinalysis blood urea nitrogen liver function tests roentgenological examinations of the chest and other structures skin tests and complement fixation tests for coccidioidomycosis were obtained prior to the initiation of therapy and at appropriate intervals during the course of therapy

Results

TABLE 2 summarizes the results of the treatment of these patients with orally administered pimaricin Improvement with the concomitant administration of pimaricin occurred in only one patient whose case history is detailed below

Case 2 R D 32 year old male Negro with disseminated coccidioidomycosis previously treated with amphotericin B with apparent cure suffered a relapse manifested by a granulomatous lesion on the right side of the neck The patient was given pimaricin in doses ranging from 200 to 1000 mg per day for a period of 154 days to a total dose of 111 2 gm During this time his albumin globulin ratio reverted toward normal changing from 2 9/5 0 to 3 4/4 2 His hemoglobin dropped from 12 0 to 7 4 but it was subsequently discovered that the patient had bleeding hemorrhoids and with their control and iron therapy the patient s hemoglobin returned to normal while he was still receiving pimaricin The white count platelets and reticulocytes as well as the BUN and creatinine remained unchanged No evidence of any abnormalities occurred in the urine During this time however his complement fixation titer for coccidioidomycosis dropped from 1 2048 to 1 64 The patient experienced mild diarrhea which became more severe together with nausea and vomiting during the first 2 days of treatment These symptoms disappeared upon temporary discontinuance of the drug and thereafter the only side effect was mild diarrhea as long as the dosage of the drug was maintained below 800 mg daily Nausea anorexia and moderately severe diarrhea occurred consistently when the daily dose of the drug was increased to more than 800 mg Two months following the institution of therapy the granuloma was completely healed However 5 months following discontinuance of therapy evidence of a granuloma occurred in the same area Therapy with pimaricin was reinstituted with a daily dose of 400 mg This was continued for a period of 6 months and resulted in complete healing of the granuloma of the neck but only partial regression of a nearby enlarged regional lymph node The total dose that this patient received with no evidence of laboratory or serious clinical toxicity was 183 2 gm

In addition two other patients (cases 5 and 9 TABLE 2) showed some improvement during treatment

Case 5 S D 36-year-old male Negro with disseminated coccidioidomycosis manifested by a coccidioidal osteomyelitis of the right wrist demonstrated apparent early healing and union of a previously chronic nonunion of the wrist but subsequently relapsed despite continued treatment

Case 9 D C, a 3½ year old white female with disseminated mucocutaneous candidiasis, showed early evidence of improvement, with partial clearing of facial and mucosal lesions and apparent healing of two fingernails However, the duration of therapy and observation has been too short to allow any conclusions to be drawn at this time

Toxicity

No laboratory evidence of toxicity was observed in any of these patients. Clinical evidence of toxicity consisted of varying degrees of anorexia, nausea, vomiting, flatulence and diarrhea These signs were of such severity as to be the major limiting factor in ascertaining total daily dosage that could be administered This was especially true in those instances where maintenance of adequate nutrition was essential to the patient s welfare

In an effort to explore the possibility of administering pimaricin intramuscularly, 0 25 mg in 0 5 cc of saline was injected into the buttocks of patient 5, resulting in a painful tumescence at the site

Discussion

The oral administration of the currently available preparations of pimaricin in dosages acceptable to the patient appeared to have little influence on the clinical course of several varieties of mycoses The one apparently good result concomitant with the administration of pimaricin occurred in a patient with disseminated coccidioidomycosis a disease characterized by the occasional occurrence of spontaneous remissions The therapeutic value of pimaricin in this patient must be interpreted with considerable reservation especially in view of the fact that the other six patients with coccidioidomycosis who were treated in this series demonstrated no improvement

The major limiting factor in the oral administration of pimaricin has been the nausea vomiting, and diarrhea that have developed regularly when dosages in the range of 600 mg to 1000 mg per day in the adult have been reached Thus far we have been unable to ascertain whether this represents direct local action on the gastrointestinal tract or central toxicity following absorption Although several antinauseant and antiemetic drug were administered during therapy, no consistent beneficial effect was obtained

The administration of pimaricin intramuscularly in a dose 0 25 mg in 0 5 cc of saline was followed by an extremely tender tumescence indicating that this route of administration would not provide a means by which therapeutic amounts of the drug could be routinely administered Preliminary studies are now in progress to evaluate the intravenou route of administration

Summary

Pimaricin was administered orally to ten patients with a variety of systemic mycoses, including patients with disseminated coccidioidomycosis disseminated mucocutaneous candidiasis and a mycetoma due to *Monosporium apiospermum*

Nausea vomiting and diarrhea limited the use of this drug in the adult to dosage levels of 600 to 1000 mg per day

One patient with disseminated coccidioidomycosis improved significantly during this therapy, but this must be interpreted with a great deal of reservation

Tentatively in its present form and in doses acceptable to the patient pimaricin does not offer promise as an effective oral antifungal agent for the treatment of systemic mycoses

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GRISEOFULVIN

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The antifungal antibiotic griseofulvin represents a major advance in the treatment of superficial fungus infections due to dermatophytes It is a highly distinctive agent among the antibiotics in that it specifically localizes and concentrates in the keratinized cells of the skin hair and nails Dermatophytes parasitizing these structures are inhibited in further growth and penetration and are subsequently removed by desquamation or are replaced by tissues free of infection This new mechanism of chemotherapy may provide a basic rationale for designing methods of treatment for other microbial diseases of these tissues

Griseofulvin is a metabolic product of several species of the genus *Penicillium* It was first isolated from homogenized mycelium of *P griseofulvum* by Oxford *et al* in 1939[1] The failure of the compound to demonstrate activity against bacteria resulted in no further study of its properties by those investigators Brian and his co workers in 1947[2] recovered a metabolite from cultures of *P janczewski* that produced hyphal abnormalities in the fungus *Botrytis allii* This compound was termed the curling factor and a year later Grove and McGowan[3] showed it to be identical with griseofulvin The chemical structure of the antibiotic was established by Grove *et al*[4] The compound is a white bitter neutral thermostabile substance chemically designated as 7-chloro 2 4,6 trimethoxy 6 methylspiro[benzofuran 2(3H) 1 (2)cyclohexene] 3 4' dione It is weakly soluble in water (0 001 per cent) but soluble in ethanol, methanol (0 1 per cent) and dimethylformamide (12 0 per cent) Its thermostability is attested to by the fact that it remains fully potent in the dry state for at least 2 years at a temperature of 39° C and can be autoclaved at neutrality for 30 min or more without loss of activity The systemic antimycotic activity of griseofulvin in plants was shown by Brian and co workers in 1951[5] The compound has been widely used in agriculture to control and treat *Botrytis* infection of lettuce and *Alternaria* blight of tomatoes

The first report of the value of griseofulvin administered by the oral route for the treatment of experimental dermatophyte infections of animals was made by Gentles in 1958[6] Guinea pigs infected with *Microsporum canis* were cured with an oral dose of 60 mg/kg of the antibiotic The drug was administered 10 days after infection and marked clinical improvement was noted within 4 days of therapy At the eighth day of treatment the hair follicles were largely free of invading fungal structures and the hair formed during the treatment period was completely resistant to invasion by the dermatophyte Similar success was observed when the animals were experimentally infected with *Trichophyton mentagrophytes* The preliminary findings of Gentles were confirmed by Martin[7] who demonstrated that experimental dermatomycoses in guinea pigs can be controlled by oral doses of 25 to 100 mg kg of griseofulvin Topical application of 1 per cent griseofulvin ointment inhibited the development of skin lesions when applied on the fourth day after infection Topical

griseofulvin in the treatment of tinea capitis and other dermatophytoses in children proved ineffective in studies by Peterkin[8] and Pardo Costello[9] More recently Goldman *et al*[10] report that a lotion containing 1 5 per cent griseofulvin with surfactants stabilizer and urea in glyceride oil proved to be highly effective in clearing affected areas in patients with tinea capitis t corporis t cruris and t pedis In a recent study *Gentles*[1] *reported that 8 daily* doses of 15 mg kg halted the progress of infection however, reinfection from fungal elements in the distal portions of the hair occurred after cessation of treatment

Orally administered griseofulvin was employed successfully by Lauder and O Sullivan[2] to treat *T verrucosum* infections in cattle Kaplan[13] treated cats spontaneously infected with *M canis* at a daily dosage level of 40 to 60 mg /kg Clinical cures were obtained in all animals within a maximum of 5 weeks of therapy Inasmuch as cats consitute the major source of human infections by the species *canis* griseofulvin can be used to control this animal reservoir

The presence of active griseofulvin in the hair of guinea pigs given an oral dose of 30 mg /kg for 23 days has been reported by Gentles and his co workers[14] The hair contained approximately 6μg of the antibiotic per gram of hair Since half of the griseofulvin was extracted from the hair by cold water and the remainder by hot methanol these workers concluded that the antibiotic is not chemically bound to the keratin but occurs mainly as a simple deposit The transport of the antibiotic depot outward by subsequent hair growth blocks further invasion by the fungus and the parasite is eventually eliminated by removal or loss of the infected portion of the hair

With the publication of Gentles initial work demonstrating the effectiveness of gri eofulvin *in the treatment of experimental dermatomycoses in animals* a number of clinical groups began administering the drug to infected human beings These studies were undertaken nearly simultaneously in the fall of 1958 by three widely separated teams Williams group[15] in London England Blank and Roth[6] in Miami Fla and Riehl[7] in Vienna Austria Their highly encouraging reports as well as those from others initiated world wide interest in the new antibiotic and prompted many clinical and laboratory investigations Numerous reports of further successes with the drug began to appear in diverse publications and the present medical literature attests to the great interest in griseofulvin Information on the clinical and laboratory aspects of griseofulvin therapy accumulated so rapidly as to make possible the International Symposium on Griseofulvin held by the University of Miami Miami Fla in October of 1959 approximately one year after the first reports of its efficacy in the treatment of dermatomycoses in human beings

At the present time gri eofulvin has emerged as the treatment of cho ce in tinea corporis due to *T rubrum* tinea capitis due to *M audouini* and *T tonsurans* and other species tinea cruris with *Epidermophyton floccosum* as well as dermatophytoses due to less common species Chronic cases of tinea pedis due to *T mentagrophytes* have responded less successfully and represent the most resistant infections *in therapy* The antibiotic is still in the assessment stage and more information must be obtained before the drug can be evaluated fully

The dosage of griseofulvin currently recommended for adults is 1 0 gm per day The duration of therapy is related to the site and nature of the infect on

Lesions confined to the skin, exclusive of those occurring on the palms or soles, require 3 to 5 weeks of daily doses of griseofulvin Due to the greater thickness of the stratum corneum on the palms and soles, a longer term of treatment is necessary It would appear that clinical and mycologic cures can be anticipated after 6 to 8 weeks of griseofulvin therapy However, resistant cases may require longer periods of treatment, higher dosage levels, and concomitant topical therapeutic measures Hair and nail infections must be treated until normal structures have replaced the infected ones, or diseased portions can be removed or cut away A number of excellent studies dealing with the clinical aspects of griseofulvin therapy have been published[18, 19, 20, 21]

The widespread oral administration of griseofulvin has shown it to be a drug of remarkably low toxicity There have been no reports of significant toxic manifestations An occasional patient reports mild gastrointestinal upset, diarrhea, and headaches Drug rashes, of vesicular, urticarial, and maculo papular forms have been seen Cross sensitivity with penicillin has not yet been recorded even in patients with known penicillin sensitivity Griseofulvin therapy has not been shown to affect the trichophytin skin test reaction except to reduce the symptoms associated with a strong positive reaction No abnormalities of liver function, kidney function or blood chemistry have been observed[16] The marrow and peripheral blood counts have shown no deviations that could be attributed to griseofulvin[19] Paget and Walpole[22] reported that the intravenous and intraperitoneal administration of the antibiotic to animals in doses in excess of 50 times that employed in man inhibits mitosis of actively dividing cells, especially in spermatogenesis For this reason periodic sperm counts have been carried out on patients receiving griseofulvin Blank and Roth[19] conducted sperm studies on 15 patients in their series and none showed significant depression or deviations from the normal In a more elaborate study of the effect of griseofulvin on spermatogenesis and semen quality, MacLeod[23] observed no adverse effects in 22 normal subjects on a daily dose of 2 0 gm for 6 months The semen was examined at weekly intervals and included in the study were sperm counts motility studies and examinations of sperm morphology In addition testicular biopsies were taken from 8 men after 12 weeks of treatment

Development of strains of dermatophytes resistant to griseofulvin *in vitro* has been reported by Robinson *et al*[20] In this study the fungi were transferred successively on solid media containing increasing concentrations of the antibiotic to a maximum of 15 μg/ml Strains of *T mentagrophytes*, *M canis*, and *M audouini* developed marked resistance to the drug Concomitant changes in colonial characteristics and microscopic morphology were associated with the increased tolerance to griseofulvin Aytoun and his co-workers[24] also have reported success in inducing *in vitro* resistance to griseofulvin in dermatophytes Of the species studied, strains of *M canis* were found to develop the highest degree of tolerance The mechanism of resistance appears to be founded in the production of fungal enzymes which reduce griseofulvin to a biologically less active compound

As yet, no isolation of a resistant strain from patients undergoing griseofulvin therapy has been reported Infection of animals with experimentally produced resistant strains of dermatophytes has not been shown to be

recalcitrant to griseofulvin therapy.[25] A limited number of cases of dermatomycoses that failed to respond to the recommended dosages of the antibiotic has been observed. Of the fungi isolated from these cases none tested has been shown to be more resistant than the pretherapy isolate or other representatives of the species. It is postulated that these therapeutic failures find their origin in inadequate absorption of the antibiotic, inadequate dosage levels or failure of the drug to be incorporated into the keratinized tissue elements.

The incorporation of griseofulvin into a medium in which a dermatophyte is growing produces abnormalities in the resultant mycelium. Concentrations of the antibiotic as low as 0.05 μg./ml. cause excessive production of lateral branches and clubbing of the advancing hyphal tips. At higher concentrations (0.1 to 0.3 μg./ml.) the cytoplasm becomes highly vacuolated and large lipid droplets make their appearance. The mycelium develops contortions, curls and swellings, and numerous septa forms. A progressive inhibition in the rate of linear growth is observed as the concentration of griseofulvin is increased until further extension of the peripheral hyphae is halted.

The effects of griseofulvin on the internal structure of dermatophytes, as revealed by electron microscopy, have been studied by Blank *et al.*[26] The antibiotic produces drastic changes in the organization of the cytoplasm and many of the intracytoplasmic structures observed in normal cells are apparently destroyed. The complex membranous organization of the endoplasmic reticulum is disrupted and the mitochondria largely disappear. Large spherical osmophilic lipid bodies appear and replace much of the cytoplasmic content. The cells of the mycelium are greatly enlarged and balloon-like swellings are common. In these areas the cell wall is greatly thickened and in places may be three to four times normal thickness. The disruption in the internal organization of the cells by griseofulvin would indicate that the effect of the antibiotic is more fundamental than mere interference with synthesis of the chitin component of the cell wall, as had been suggested by earlier workers.[27]

In a study of the *in vitro* spectrum of activity of griseofulvin, Roth *et al.*[28] observed that the 13 recognized species of dermatophytes all demonstrated a high degree of sensitivity to the antibiotic. The minimal inhibitory concentrations of the drug after 72 hours of incubation ranged from 0.18 μg./ml. in the case of *T. rubrum* to 0.42 μg./ml. for *M. audouini*. The effect of the antibiotic on these fungi is limited to fungistasis regardless of the concentration of griseofulvin in the medium. Pellets of dermatophytes can withstand 30 days of contact with 30 μg./ml. of griseofulvin in liquid medium without loss of viability. Etiological agents of cryptococcosis, sporotrichosis, chromomycosis, *nocardiosis, blastomycosis, geotrichosis*, histoplasmosis, and coccidioidomycosis were insensitive to high concentrations (25 μg./ml.) of the antibiotic at both 28° C. and 37° C. The growth of the yeast *Candida albicans* was neither inhibited nor enhanced by griseofulvin, and the intraperitoneal injection into white mice of the yeast in combination with the antibiotic had no potentiative effect on its pathogenicity such as is observed when tetracyclines are so employed. Griseofulvin in high concentration did not affect the growth of a select group of 18 species of pathogenic bacteria. Similarly, 40 species of nonpathogenic ascosporogenous yeasts and imperfect yeasts and yeastlike fungi were completely resistant to a high concentration of the antibiotic.

Emmons[29] tested the effect of griseofulvin on mice experimentally infected with the agents of histoplasmosis, blastomycosis, cryptococcosis, and coccidioidomycosis. The antibiotic was administered orally, intraperitoneally, and intravenously in daily doses of up to 30 mg per mouse. In no case did the treatment eliminate the infection or extend the survival times of the infected mice. Latapi[30] recently reported the efficacy of griseofulvin therapy in human cases of sporotrichosis and mycetomas due to *Nocardia brasiliensis*. The antibiotic was administered orally in daily doses of from 1 to 2 gm. per day. Favorable responses, even to clinical cures, were observed in these mycotic diseases in spite of the lack of *in vitro* sensitivity of the etiological agents to griseofulvin. No evidence of improvement was noted in cases of chromomycosis similarly treated.

The presence of griseofulvin in human stratum corneum and its rate of transport through the epidermis has been demonstrated by Roth and Blank.[31] A direct *in vivo* bio-assay procedure was employed in which macroconidia of *M. gypseum* were applied to the skin of the inner surface of the forearm in microslide ring cultures. The presence of the antibiotic in the stratum corneum was established by the induction of morphologic abnormalities in the emerging germ tubes of the spores. Griseofulvin does not inhibit germination of the macroconidia but results in the clubbing, contortion, and branching of the primary hyphal elements. The progress of griseofulvin through the thickness of the stratum corneum was determined by stripping off the cornified cellular elements to the desired depth with multiple applications of pressure sensitive tape. The antibiotic was detected at the junction of the stratum corneum and stratum mucosum in from 48 to 72 hours after onset of therapy. In subjects receiving 1 gm. of griseofulvin daily, the drug had been transported through the 25 per cent level of the stratum corneum in from 7 to 12 days and attained the midpoint in from 11 to 19 days. The time required for the transport of the antibiotic to the various levels of the epidermis was directly correlated to the thickness of the horny layer of the subject. Utilizing this bio assay technique, no unequivocal evidence of the presence of active griseofulvin at the surface of the intact skin was obtained even though some subjects were on therapy for as long as 7 weeks.

In spite of its limited solubility in water, the antibiotic is quickly absorbed from the gastrointestinal tract. Employing spectrophotofluorometric assay techniques, Child and Tomich[32] showed that peak blood levels occurred 4 hours after a single oral dose of griseofulvin. The compound was concentrated in the skin, liver, fat, and skeletal muscle. In man and rats the half life of the antibiotic during the decay period was approximately 6 hours. Sixteen per cent of an oral dose was excreted in the feces and less than 1 per cent was detected in the urine in the 24 hours following administration. Experiments in which rat tissue slices were employed showed that griseofulvin was rapidly destroyed by the liver, but was not inactivated by the spleen, lung, or kidney.

As yet little is known of the mechanisms by which griseofulvin inhibits the growth of sensitive fungi. Roth and his co-workers[33] conducted preliminary studies on the effect of the antibiotic on the respiration of *T. rubrum*. Utilizing Warburg manometric determinations, these investigators showed that griseofulvin had no effect on the cytoplasmic energy centers of the fungal cell. They

have postulated that, if energy mechanisms are influenced by the drug, it must be at a superficial locus such as the cell wall area Recent studies by McNall[illegible] have shown that the inhibitory effect of griseofulvin is based, at least in part, on an interference in the synthesis of fungal nucleic acids The antibiotic within the cell competes with purine nucleotides essential for elaboration of nucleic acids The competitive inhibitory action is not complete, and the effect of the drug can be reduced or reversed by normal metabolites Griseofulvin thus retards the metabolic activities of the fungus and curtails growth to a degree sufficient to permit the host to eliminate the arrested organism by exfoliation of the stratum corneum

The availability of a highly efficacious and specific chemotherapeutic agent for the treatment of dermatophytoses imposes a greater responsibility on both the clinician and the medical mycologist Since clinical manifestations of candidiasis, psoriasis bacterial infections, alopecias, and other dermatologic disorders often closely resemble those of the superficial mycoses, it is mandatory that competent mycologic studies be performed Consideration of the prolonged course of griseofulvin therapy frequently required in onychomycosis and other chronic tineas makes definitive laboratory studies of paramount importance In the final analysis, the only criterion that can determine the required dosage levels and duration of treatment with griseofulvin and the final attainment of a biological cure are the laboratory findings obtained by the medical mycologist

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GRISEOFULVIN IN DEEP MYCOSES

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During the recent symposium on griseofulvin held in Miami, Fla., in October 1959,[1] the agreement was reached that this antibiotic is useful only in dermatophytoses or mycoses caused by dermatophytes, a fact that had been foreseen from its fungistatic spectrum. It was also the consensus that the antibiotic is almost atoxic, and that it does not produce resistance *in vivo*.

Among the points on which a difference of opinion did exist is that of griseofulvin's activity in deep mycoses.[1,2] The experiences of Emmons[3] on the failure of griseofulvin in the experimental infections of mice by *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides immitis*, and *Blastomyces dermatitidis* were in accordance with the results *in vitro*. Notwithstanding this definite information my associates and I treated some patients with deep mycoses using griseofulvin and taking into account the fact that there have been previous experiences in microbiology where there is a lack of parallelism between results *in vitro* (or even in experimental infection) and clinical results.

The patients with deep mycoses subjected to treatment with griseofulvin were four with actinomycotic mycetoma from *Nocardia brasiliensis*, one with severe primary pulmonary histoplasmosis, two with chromoblastomycosis, and two with sporotrichosis. Except in treating sporotrichosis, griseofulvin failed as a therapeutic agent in all of these patients. The data from these observations constitute the material of this report.

Actinomycotic Mycetoma

In all 4 adult patients mycetoma was due to *N. brasiliensis* and was localized in the lower extremity. The duration of the infections varied, the extremes being a case that lasted 6 months, in which only the soft tissues were affected and another that had resisted all treatment for 8 years, this case involved infections of bones of the leg and pelvis, and also of the urinary bladder. Griseofulvin was given to all 4 patients, the daily dose of 1 gm. being divided into 4 administrations. This treatment was usually maintained for 2 months and then suspended for lack of noticeable improvement.

Chromoblastomycosis

Two of these patients were adults, both infected by *Hormodendrum pedrosoi*, the first had a foot infection that had lasted 4 years, the second suffered infection of the foot and leg with a 6-year long evolution. The latter patient had undergone surgical removal of diseased skin with the consequent skin grafts but the verrucous lesions reappeared in 3 places with abundant parasites. Since the administration of griseofulvin did not improve the clinical picture it was suspended after the usual 2 months.

Histoplasmosis

A young Swiss stayed in México, D. F., for a few months then visited a cave near the city of Orizaba in the state of Veracruz. Twelve days later he

presented fever and severe dyspnea, X rays showed abundant nodular infiltrates in both lungs, precipitin test with histoplasmin was positive at 1:32 dilution, and *H. capsulatum* was observed in sputum and was later isolated by culture. A slight improvement was observed after 1 month of development, then fever and dyspnea increased, and X rays showed confluence of the nodules, especially in both bases. This was probably due to the previous administration of ACTH in order to decrease the temperature. Under these circumstances treatment with griseofulvin was started at 2 gm/day in 4 administrations. The patient's condition deteriorated and, after 35 days, treatment was suspended; he died 10 days later.

Sporotrichosis

We treated an outbreak of sporotrichosis in 3 patients exposed to the same focus of infection: a father, a mother and their son, all of whom showed cutaneous lesions at the same time. The father, 32 years old, and the mother, 24 years old, presented lymphangitic type sporotrichosis with gummatous developments[4] on the right arms (FIGURES 1a and b); the child, 8 months old, presented a fixed type sporotrichosis with a verrucous clinical form[4] on the left temporal region (FIGURE 1c). After 2 months of clinical course the 3 patients were treated as follows: the father with potassium iodide, beginning with 0.50 gm and increasing the same amount per day until the daily dose was 4 gm. This dosage was continued over a period of 3 months. Both mother and son were subjected to treatment with griseofulvin: the mother received 1 gm and the son 250 mg daily divided in 4 administrations for a period of 3 mos. The results in all 3 cases were amazingly similar. None of the patients presented any noticeable change after 2 weeks of treatment. One month later a 30 per cent improvement was observed in all three. After 2 months the clinical improvement was estimated at 70 per cent. The patients were considered cured 3 months later and therapy was suspended. Only keloid scars remained, less noticeable in the child (FIGURES 1d, e and f). The patients continued under observation for 5 months after treatment was suspended and no relapse was observed. The antibiotic produced no side effects.

The interest of this report lies perhaps in the fact that in sporotrichosis the same phenomenon is observed in relation to griseofulvin as to potassium iodide, that is, there is no parallelism between its action *in vitro* and its effect *in vivo*; this is true to such an extent that a 10 per cent concentration does not inhibit the growth of the organism[7] and the substance does not prevent experimental sporotrichosis.[6] Consequently, there is still no convincing explanation for the action of potassium iodide and griseofulvin in infections by *Sporotrichum schenckii*. Since cutaneous sporotrichosis has a specific treatment in potassium iodide, for obvious reasons griseofulvin would in no way be a substitute for it, but knowledge of its usefulness in cutaneous sporotrichosis authorizes its use in visceral forms, as in pulmonary sporotrichosis, in which potassium iodide does not achieve a cure. It is also possible that synergism may exist when both substances are used.

Summary

Observations are presented on the results of treatment with griseofulvin in 4 patients with actinomycotic mycetoma from *N. brasiliensis*, 2 cases of chromo-



FIGURE 1

blastomycosis 1 case of severe primary pulmonary histoplasmosis and 2 cases of sporotrichosis With the exception of sporotrichosis griseofulvin failed as a therapeutic agent in all of these patients

The 2 cases of sporotrichosis described in this study involved an outbreak in 3 patients a father a mother, and their son The father was treated with potassium iodide the mother and son with griseofulvin The beneficial results with griseofulvin were identical to those obtained with potassium iodide given to the father as control Complete cure was obtained in the same length of time with both medications 3 months of treatment for each patient The cases continued to be under observation for 5 months after treatment was suspended and no relapse was observed The antibiotic produced no collateral effects

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NYSTATIN

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Nystatin is a polyene antibiotic of broad antifungal spectrum produced by *Streptomyces noursei*.[1] The antibiotic possesses certain unique chemical and biological properties that have given it a distinctive place among antifungal agents of importance, and also certain undesirable characteristics that have restricted its potential usefulness. In this paper we discuss some of the important chemical properties, the mode of action, development of resistance, the practical applications in the laboratory, and the use in therapy and prophylaxis of fungus diseases.

Physical and Chemical Properties

The more significant physical and chemical properties of nystatin are briefly presented as a basis for better understanding of the antibiotic. One of the outstanding characteristics is its slight solubility. Except at acid or alkaline reactions, nystatin is almost insoluble in water, but it is somewhat more soluble in the lower aliphatic alcohols, especially if water is present, and it is highly soluble in solvents such as propylene glycol, N,N-dimethylformamide, or dimethylsulfoxide.

Although nystatin was the first of the so called polyene antibiotics to be reported,[2] its chemical structure has not been completely elucidated. It is known to have both a conjugated diene and a conjugated tetraene moiety in the molecule.[3] Of considerable interest in the chemistry was the identification of the aminomethyldeoxypentose, mycosamine,[4] which was also identified in pimaricin,[5] another tetraene, and in amphotericin B, a heptaene. The infrared spectrum indicates the presence of carboxyl and lactone structures. Because of the extensive unsaturation of the molecule, nystatin is unstable and hence readily inactivated. Crystalline material, however, has been stored at refrigerator temperature for 8 years without appreciable loss of activity, and aqueous suspensions were stored at −25° C for 18 mo without deterioration. At room temperature both alcohol solutions and aqueous suspensions gradually lose potency.

Mode of Action

Since nystatin is representative of a group of antibiotics specifically effective against fungi, its mode of action is naturally under study by several investigators, and some of their findings will be reviewed briefly. Lampen and his co-workers[6-8] have observed the conditions under which this antibiotic is taken up by sensitive microorganisms and have demonstrated that resistant strains of *Candida* absorb less than do sensitive strains. Since the methyl ester and the N acetyl derivatives, which are not inhibitory to fungi, are not absorbed to any significant extent, these authors postulate that the ionic species HOOC - NH_3^+ in the molecule is responsible for the absorption by the cell. Working with bakers' yeast exposed to nystatin, Lampen and his asso

ciates observed an increase in the production of triosephosphate, pyruvate, and malate and a marked reduction in activity of the following enzymes aldolase glyceraldehyde phosphate dehydrogenase, and phosphoglycerate kinase They conclude that this effect on enzymes may be due to proteolytic activities released under the influence of the antibiotic Horvath *et al*[9] further more observed that nystatin in less than fungistatic concentrations inhibited the production of amylase by *Penicillium chrysogenum*

In the light of possible released proteolytic activities it is interesting to note the following random observations with *Histoplasma capsulatum*[10] The yeastlike cells that normally, upon treatment with concentrated hydrochloric acid, yield soluble biuret positive fractions active in the fixation of complement, failed to do so if first exposed to nystatin Thus there apparently was alteration in the protein components as evidenced by lack of serologic activity This treatment resulted in shrunken cells accompanied by a decrease in the volume of packed cells

While Harman and Masterson[11] conclude that nystatin acts on several stages of glucose metabolism by *Candida albicans* and also depresses glucose uptake by alteration of cell permeability Lampen *et al*[12] found that glucose is still able to cross the cell wall of *Saccharomyces cerevisiae* rendered incapable of utilizing glucose by exposure to the antibiotic Osteux and his co-workers[13] observed increased amounts of radioactive P mainly as orthophosphate in the medium used to suspend labeled *C albicans* cells following the addition of nystatin and interpreted this to mean selective modification of cell membrane permeability Bradley[14,15] reports that the inhibition of the assimilation of glucose by *C stellatoidea* exposed to this agent is not primarily an effect on permeability He found cell wall development and protein synthesis unaltered and inhibition of glucose fermentation accompanied by altered phosphate metabolism and reversible by phosphate Studies with the electron microscope indicated to Blank[16] that nystatin induced cytolysis of *C albicans* cells without destroying the cell walls Ribereau Gayon and his co-workers[17] found that the respiratory, and especially the fermentative, activities of *Saccharomyces ellipsoideus* were inhibited by the antibiotic more strongly at pH 3 8 than at pH 7 0 when actually at neutrality small amounts enhanced fermentation

Since some of these physiological metabolic and biochemical responses to nystatin are not shown by bacteria or the occasional fungi that are resistant to the action of the antibiotic the effects may be considered as peculiar to the genus and even to the species

Development of Resistance

The possibility that resistant microorganisms may develop during therapy with nystatin has concerned many investigators Several[18-21] of these researchers have reported that recent isolates of various species of *Candida* are sensitive to small amounts of antibiotic while Roda *et al*[22] found that 36 of 79 strains of *C krusei* were not inhibited in disc tests, although other species were *C albicans* isolates obtained before and after treatment have shown the same degree of sensitivity[18,20,23] Moreover relapses following the cessation of treatment for moniliasis apparently have not been due to the presence of

resistant microorganisms *In vitro* experiments[7,28] have demonstrated that a low grade resistance of *Candida* strains may be induced, although inconsistently The mutants resistant to nystatin were also resistant to amphotericin B[5] or to sodium arsenate[31] and vice versa A significant observation was made by Manning and Robertson[30] when treating a case of pulmonary aspergillosis No resistant strain emerged after 38 days of nystatin therapy but the isolated *Aspergillus fumigatus* developed *in vitro* resistance in the laboratory It is thus obvious that the acquisition of resistance *in vitro* is not the reflection of any *in vivo* pattern and that therapy with this antibiotic is to date uncomplicated by the appearance of resistant pathogenic fungi

Laboratory Applications

In the laboratory, nystatin has found extensive applications[32-37] for the control of yeast and mold contamination in bacteriological media in samples of biological materials of human and animal origin from which the isolation of pathogens is to be attempted, and especially in tissue culture techniques Effective concentrations are not inhibitory to growing viruses and not toxic for various lines of cells from human, murine, rabbit porcine, bovine, feline, chick and monkey sources

Therapy and Prophylaxis

The therapeutic and prophylactic applications of nystatin stem from its *in vitro* antifungal properties It possesses strong activity against a wide variety of pathogenic and nonpathogenic yeasts and, to a somewhat lesser degree filamentous fungi Animals infected experimentally with lethal doses of *Cryptococcus neoformans*, the yeastlike forms of *Blastomyces dermatitidis*, *H capsulatum* and *C albicans* received marked protection from nystatin injected subcutaneously[40] No detectable cumulative systemic toxic effects resulted from repeated subcutaneous doses At the site of injection there were induration and necrosis that healed spontaneously Significant blood levels following subcutaneous injections of single doses of 100 mg /kg , were demonstrated in the mouse

These potentialities of nystatin as a therapeutic agent, suggested by its effectiveness in animals have not been entirely realized in man since the antibiotic displays toxic properties and is poorly absorbed from the gastrointestinal tract The toxic effects depend upon the route of administration Applied topically orally or by inhalations or instillations in the form of an aerosol nystatin is remarkably free of harmful reactions Few allergic effects or cases of contact dermatitis have been reported[41] Newcomer and his associates[42] observed no side reactions other than transitory nausea and diarrhea when oral treatment was prolonged for 6 months and doses reached as high as 12 gm daily Parenteral injections however, were shown in early clinical investigations to induce undesirable reactions Given intravenously, it caused sclerosing of the veins, the initial injections being accompanied by shaking chills fever, and malaise, intramuscular injections were followed by severe pain and tenderness at the site of injection The unsuitability of the parenteral routes of administration and poor absorption from the gastrointes

tinal tract have curtailed its usefulness in therapy of the deep mycoses and the dermatophytoses as well

However nystatin has proved remarkably effective in diseases where immediate direct contact between fungus incitant and antibiotic could be accomplished by topical application oral administration inhalation or local injection Favorable reports on the effectiveness of treatment of the various clinical forms of moniliasis (candidiasis) in infants children, and adults are to be found in increasing number in the medical literature Only a few of the more recent papers will be cited since many earlier ones were previously reviewed

Infections in infants involving the oral cavity gastrointestinal urinary and respiratory tracts intertriginous folds genitocrural region or cutaneous tissues have responded well as a whole often dramatically without significant side effects Response to retreatment was usually prompt in instances of relapse in a few patients there was no response to treatment In the treatment of 20 cases of oral thrush with lyophilized nystatin Kozinn and Taschdjian reported 11 infants cured after 1 course of therapy 7 relapsed but responded promptly to retreatment and 2 improved but were not cured after 5 to 7 days when they were lost to observation In addition Pohowalla and his associates found that 75 per cent of 28 infants with oral moniliasis three fourths with complications of monilial diarrhea, were clinically cured by oral nystatin by the seventh day and 60 per cent were culturally negative Seven of 13 suffering from dehydration died These authors warned that dehydration in babies with thrush carries a high mortality Huang and High found the antibiotic ineffective in 7 of 41 infants and children treated for oral moniliasis ? of these 7 were in a group of chronically ill older infants and children and premature infants many of whom were being treated with other antimicrobial agents Gratifying results with combined oral and topical applications in 40 of 46 infants with eruptions of the genitocrural area were reported by Robinson Dobias found that 4 infants with Candida in the stools and oral and cutaneous moniliasis when treated with oral nystatin experienced complete disappearance of a diarrhea due probably to gastrointestinal moniliasis symptoms of vulvovaginitis in a 2 month-old girl disappeared after 5 days of oral nystatin and an infant with cutaneous moniliasis dyspnea cyanosis and X ray evidence of pulmonary infiltration was cured of her respiratory symptoms after 3 days of oral nystatin alone Debré and his associates also found nystatin a favorable therapeutic agent in Candida infections of the intestinal mucous membrane cutaneous abscesses and infections of the urinary tract

Nystatin has also proved efficacious in moniliasis of children and adults although in some instances a long period of treatment may have been required Howell Strauch and Levine and Grupper had remarkable success with nystatin in infections of the mucous membrane skin and nail and observed no harmful effects due to this antibiotic Grupper pointed out the necessity of debriding infected foci at the base of the nails in chronic infections so that the antifungal agent could penetrate below the deeper layers of keratin where C albicans is localized and he advised as others have the concurrent use of topical and oral treatment Wright and his associates treated effectively

122 infections of the mucous membranes and cutaneous tissues with nystatin Five patients in whom the results were only fair had severe systemic illness or were receiving prolonged steroid or antibiotic therapy These investigators stated that the clinical effectiveness was influenced by the type of vehicle in which nystatin was placed For example, when it was used in ointment form to treat infections of the intertriginous areas the results were poor in contrast to rapid healing of the lesions when the drug was in solution Dobias[56] has similarly warned against the use of any ointment on moist lesions

Sahni[57] effectively treated a pulmonary moniliasis of an eight year-old child with potassium iodide and with nystatin both orally and by inhalation He stated that it would be difficult to judge the relative roles played by the nystatin and the potassium iodide but added that the duration of active therapy required was shorter than that reported when the iodide is used alone McKendrick and Medlock[58] successfully employed inhalations of the antibiotic in aerosol in pulmonary moniliasis complicating fulminating influenzal pneumonia Stewart[43] found nystatin to be an agent of very definite importance in the treatment of pharyngorespiratory mycotic infections He emphasized however that any assessment of the efficacy of nystatin against such infections must be based upon a critical realization of the superficial nature and tendency to spontaneous amelioration of many monilial lesions Robinson[59] found that the oral administration of large doses was effective in the treatment of a patient with cutaneous and urinary tract infections Employing oral nystatin Barrett and his associates[60] failed to prevent a fatal outcome of systemic moniliasis complicating pancreatitis with pancreatic abscess The patient had received broad spectrum antibiotics over a long term They suggest that the concurrent use of a fungicidal agent be considered when broad spectrum antibiotics are used in large doses for long periods of time Vanbreuseghem and Eyckmans[61] met with no success in the use of very large oral doses of nystatin in a patient with chronic moniliasis of long duration in whom local treatment with antiseptics and antifungals as well as actinotherapy had failed

Yaffe[6] employing intraperitoneal and intramuscular routes of injection of nystatin dissolved in propyl alcohol effectively treated a case of systemic moniliasis including granulomatous lesions of spleen and liver associated with prolonged use of broad spectrum antibiotics Duncan[63] using salve reported prompt success in the treatment of a cutaneous moniliasis of the axilla resulting from the daily use of a deodorant ointment containing antibiotic

Nystatin should find its greatest usefulness in monilial vulvovaginitis particularly in pregnant women Reports[6, 67] of excellent results are frequent in the current literature indicating this antibiotic to be the therapy of choice for this annoying and common infection Relief of symptoms is usually prompt and without evidence of toxicity

On the controversial subject of the prophylactic use of nystatin Welch[68] after consideration of the conditions under which moniliasis is most likely to develop concluded that logically the antibiotic should be administered prophylactically whenever high doses of the broad spectrum antibiotics and/or the concurrent use of cortisone and related compounds are required for debilitated patients diabetics and infants (particularly premature) Such situations

are frequently faced in the antibiotic treatment of bacterial endocarditis and meningitis, or for the prevention of rheumatic recurrences and in the treatment of leukemia with steroids

As a prophylactic agent nystatin should find its greatest usefulness in the prevention of neonatal thrush, with its serious consequences.[69] As early as 1938 Woodruff and Hesseltine[70] showed that the incidence of thrush in the newborn has a direct relationship to the presence of fungi in the generative tract of the mother This they found to be as high as 29 per cent in the third trimester of pregnancy and considerably higher in the lower economic groups B Dobias (personal communication) recommends a careful mycologic examination of the vagina of every pregnant woman, and the use of nystatin therapy in every patient in whom *C albicans* is found Pace and Shantz[68] have stated that the protection of an infant against exposure to a potentially dangerous *C albicans* infection demands the prompt and effective treatment of every pregnant patient with vaginal moniliasis, they recommend prophylactic use of nystatin during the last week or 10 days of pregnancy as a justifiable and desirable measure in these women Harris and his associates[71] endeavoring to eliminate thrush in the newborn nurseries found that of 714 infants treated with nystatin not 1 developed thrush during its hospital stay and only 3 developed it within 1 week after discharge In the control group of 728 untreated infants 18 developed thrush in the hospital and 13 in their first week at home These investigators concluded that thrush can be eliminated completely from hospital nurseries by the simple harmless and inexpensive procedure of instilling nystatin solution into the mouths of new born babies

Other mycotic infections in which nystatin therapy appears to offer promise are aspergillosis of lungs eye and external ear and accessible lesions of histoplasmosis.

Riddell[72] reported early that inhalation of nystatin in suspension was effective in removing *A fumigatus* from bronchial secretions Manning and Robertson[73] successfully treated a secondary infection due to *A fumigatus* in a long standing pyopneumothorax The fungus was isolated in pure culture on 3 occasions from the sputum and once from the intrapleural cavity before treatment was begun Nystatin was introduced in aqueous suspension by intrapleural installations at first on successive and later on alternate days for 38 days No side effects were noted The authors thought it probable that *A fumigatus* established itself in the pyopneumothorax as a result of treatment with a broad spectrum antibiotic over a period of 16 days

Gero and Szekely[74] reported success in the treatment of a pulmonary aspergillosis by introduction of nystatin in aerosol containing 30 000 to 500 000 U thrice daily with interruptions of 1 to 2 days At the end of 4 weeks the general condition of the patient had improved After further treatment of 6 months the radiograph had cleared the sputum was negative for *Aspergillus* and the patient was back at his profession Prior to treatment sputum and bronchoscopic wash fluid had shown the presence of fragments of hyphae on direct films and the culture produced *A fumigatus*

Mangiaracine and Liebman[75] reported that nystatin exerted a beneficial

effect in the treatment of a case of hypopyon keratitis caused by *A fumigatus* they employed oral doses in conjunction with instillations of an aqueous suspension into the conjunctival sac

By injection of nystatin directly into oral *Histoplasma* lesions, Plotnick and Cerri[75] brought about complete disappearance of the localized disease process *H capsulatum* was found in histological sections of biopsy of the lesion and was isolated from the specimen before treatment

Summary

Nystatin is a valuable therapeutic and prophylactic agent for the many clinical forms of moniliasis and has been shown to be efficacious in the treatment of pulmonary aspergillosis

This antibiotic has a broad antifungal activity, its chemical properties are unusual it is not known to induce *in vivo* resistance, and it has been shown to induce only a low grade resistance *in vitro* It lacks oral and topical toxicity

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TENNECETIN

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Tennecetin, an antifungal antibiotic, was isolated by Burns and Holtman[1] from strains of *Streptomyces chattanoogensis* obtained from Tennessee soil.

Characteristics of the Organism

One of the most prominent characteristics of the organism is its ability to produce a deep yellow-orange pigment in most media. On solid media the pigment diffuses into the agar. The vegetative mycelium of the organism itself is of orange yellow color.

Sporulation occurs only on certain media, for example, Carvajal's oatmeal agar. Microscopically, spores are found in chains at the ends of coiled sporophores. Other characteristics are: gelatin is rapidly liquefied; starch is hydrolyzed; milk is coagulated and peptonized; hydrogen sulfide is not produced; growth without sporulation occurs at 37° C., but not at 45° C.; growth is inhibited in media containing 3 per cent sodium chloride; and a yellow ring often appears at the surface of litmus milk tubes, but a true pellicle has not been observed on any liquid medium.

It was not possible to identify this organism with any of the previously described species of streptomyces.

Production and Recovery of Tennecetin

For production in quantity, a shaken broth culture of the organism is inoculated into fermenters equipped with mechanical agitators, filled with GPY media[1] and aerated with oxygen during the fermentation period at temperatures of 25 to 28° C.

Unlike most other polyenes, tennecetin occurs almost entirely in the culture liquid. For this reason, the mycelia are filtered off before extracting the antibiotic. Extraction is accomplished with *n*-butanol, followed by concentration of the butanol extract *in vacuo* and precipitation of the active material by addition of four volumes of anhydrous ether.

The product resulting from this procedure is a yellow amorphous powder containing up to 100 U. of tennecetin activity per mg. of dried powder.[1]

Assay of Tennecetin

A quantitative microbiological assay method has been developed for measuring the amount of tennecetin in crude broths, extracts, and dried powder. This is the paper disc agar diffusion method, using *Saccharomyces carlsbergensis* as the test organism. This assay method gives a straight line response with a good slope over a tenfold range in concentration when plotted on semilogarithmic paper.

Using this method, an arbitrary unit of tennecetin has been defined as that amount of antibiotic which, when contained in 1.0 ml. of broth or diluent, will give a 20 mm. zone (diameter) of inhibition against the test strain of *S. carls-*

bergensis (Strain K 20) when tested in accordance with the conditions prescribed for the standard assay method [1]

Chemical and Physical Properties of Tennecetin

Tennecetin is soluble in water, methanol, 95 per cent ethanol, propylene glycol, pyridine, formamide, dimethylsulfoxide, ethyl stearate, and *n* butanol It is insoluble in chloroform ethyl acetate, amyl acetate, ether, acetone, tetrahydrofuran, butyl stearate, methyl lineolate, and petroleum ether Aqueous solutions of the antibiotic have an alkaline reaction[1] (see also F S Barr, personal communication)

Solutions of tennecetin kept in the refrigerator have retained full activity for more than 1 month At *p*H 7 0 solutions of the antibiotic have retained full activity following treatment at 100° C for 20 min Stability is markedly decreased at *p*H 4 0 and *p*H 10 0 Solutions of tennecetin are quite insensitive to visible light [1]

All active preparations of tennecetin have exhibited a characteristic ultraviolet absorption spectrum with a slight shoulder at 270 to 280 mμ, and peaks at 288 300 to 302 and 315 to 318 mμ [1] Such an absorption spectrum is characteristic of polyene compounds in general and tetraenes in particular [2]

When compared with the published spectra of other tetraenes, the infrared absorption of tennecetin shows several variations in the major and minor absorption bands of all indicating that they are not identical compounds [1]

Paper chromatography of tennecetin showed R_f value of 0 33 (as compared to R_f of 0 38 for Rimocidin sulfate and R_f of 0 22 for nystatin) [1]

Biological Properties of Tennecetin

Tennecetin has been found to be markedly fungistatic or fungicidal, or both, to yeasts and filamentous fungi *in vitro* More than eighty different species of microorganisms were tested for their susceptibility to the action of tennecetin Thus far, no yeast or mold has been found that has not been inhibited by this antibiotic, although certain of the human dermatophytes have required somewhat higher *in vitro* concentrations to demonstrate the inhibitory effect Most bacteria appear to be resistant to the action of tennecetin [1] TABLE 1 lists some of the species that have been tested for *in vitro* sensitivity to tennecetin by cross streak agar diffusion technique [1]

TABLE 2 shows the amounts of tennecetin required, in units, for inhibition of some of the strains tested by Burns and Holtman [1]

TABLE 3 shows the results of similar tests done in the Laboratory of Mycology, College of Medicine, University of Cincinnati (J Schwartz, personal communication)

No growth of any of the organisms tested occurred in the media containing the various concentrations of tennecetin employed

Toxicological Studies

The acute and chronic toxicity of tennecetin for 6 species of laboratory animals has been reported by Barr [3] The agent was found to be toxic when

TABLE 1

SENSITIVITY OF MICROORGANISMS TO TENNECETIN BY CROSS STREAK AGAR DIFFUSION TECHNIQUE

Organisms inhibited	
Penicillium sp	*Corynebacterium* sp
Aspergillus sp	*Corynebacterium diphtheriae*
Aspergillus niger	*Pseudomonas tabaci*
Absidia spinosa	*Candida albicans*
Mucor sp	*Candida krusei*
Syncephalastrum racemosum	*Candida parakrusei*
Cunninghamella sp	*Candida stellatoidea*
Thamnidium elegans	*Geotrichum* sp
Circinella tenella	*Cryptococcus neoformans*
Penicillium citrenum	*Torulopsis* sp
Aspergillus candidus	*Rhodotorula* sp
Aspergillus glaucus	*Saccharomyces cerevisiae*
Aspergillus fumigatus	*Saccharomyces carlsbergensis*
Aspergillus clavatus	*Saccharomyces fragilis*
Aspergillus ochraceus	*Hansenula anomala*
Paecilomyces sp	*Hansenula mrakii*
Scopulariopsis sp	*Hansenula silvicola*
Fusarium graminum	*Pichia membranefaciens*
Fusarium sp	*Schwanniomyces* sp
Penicillium canescens	*Trigonopsis variabilis*
Blastomyces dermatitidis (yeast phase)	*Mycoderma* sp
Blastomyces dermatitidis (mycelium phase)	*Debaromyces globosus*
Sporotrichum schenckii	*Sporobolomyces salmonicolor*
Trichophyton mentagrophytes	*Zygosaccharomyces lactis*
Microsporum gypseum	*Candida tropicalis*
Microsporum audouini	
Organisms slightly or questionably inhibited	
Oospora lactis	*Sarcina lutea*
Staphylococcus aureus	*Corynebacterium* sp
Staphylococcus sp	*Mycobacterium* sp (ATCC 607)
Organisms not inhibited	
Escherichia coli	*Bacillus cereus*
Serratia sp	*Bacillus subtilis*
Salmonella typhosa	*Bacillus graveolus*
Paracolobactrum sp	*Neisseria catarrhalis*
Pseudomonas fragii	*Neisseria perflava*
Pseudomonas fluorescens	*Mycobacterium avium*
Alcaligenes viscosus	*Streptomyces* sp
Pseudomonas aeruginosa	*Nocardia* sp
Salmonella enteritidis	*Nocardia asteroides*
Bacterium cadaveris	*Micromonospora* sp

used by injection by the intravenous intramuscular intraperitoneal and subcutaneous routes The acute LD_{50} by the oral route was not reached when doses 10 times the toxic intravenous dose were used

Chronic toxicity studies done on several groups of animals with doses of 100 200 and 250 U of tennecetin daily by the oral route for periods as long as

6 months showed no toxicity during the studies. At the end of the studies only 2 animals, when sacrificed, revealed pathological changes in the vital organs. One rat, after receiving daily dosages of 100 mg. of tennecetin orally

TABLE 2
CONCENTRATION OF TENNECETIN REQUIRED TO INHIBIT GROWTH OF CERTAIN FUNGI IN VITRO*

Organism	Tennecetin U./ml. medium			
	0.5	0.25	0.05	None
Sporotrichum schenkii	0	4	4	4
Cryptococcus neoformans	0	4	4	4
Candida albicans	0	0	4	4
Candida parakrusei	0	0	4	4
Debaryomyces globosus	0	4	4	4
Saccharomyces cerevisiae	0	0	4	4
Geotrichum candidum	0	4	4	4
Saccharomyces carlsbergensis	0	2	4	4
Allescheria boydii	0	0	4	4
Trichophyton rubrum	0	0	4	4
Microsporum audouini	0	3	3	4
Blastomyces dermatitidis (yeast phase)	0	0	4	4
Blastomyces dermatitidis (mycelial phase)	0	0	2	2
Coccidioides immitis	0	0	1	2

* Tennecetin was dissolved in Sabouraud's dextrose agar. Numerals in table refer to extent of growth judged by arbitrary scale between 0 and 4 (0 indicates no growth, 4 indicates heavy growth).

TABLE 3
CONCENTRATIONS OF TENNECETIN REQUIRED TO INHIBIT GROWTH OF CERTAIN FUNGI IN VITRO

Strains	Controls	Tennecetin mg./ml.* medium†					
		5	2.5	1.25	0.62	0.31	0.16
Penicillium sp.	+	−	−	−	−	−	−
Candida albicans strain 7	+	−	−	−	−	−	−
Aspergillus sp.	+	−	−	−	−	−	−
Histoplasma capsulatum	+	−	−	−	−	−	−
Trichophyton mentagrophytes	+	−	−	−	−	−	−
Cryptococcus neoformans	+	−	−	−	−	−	−

* 1 mg. = 15.8 U. tennecetin.
† Sabouraud's dextrose agar.

for 6 months, had a hemorrhage in one lung. This hemorrhage was thought to be due to bacterial infection since it was confined to one lobe of one lung. One cat, receiving daily oral doses of 250 U. of tennecetin for ... when sacrificed, had an abnormal deposit of a ... to be fat in the liver. It was not possible to ... al stains (J. Shapiro, personal communicatio

Systemic in Vivo Studies

The use of tennecetin by parenteral routes is apparently not practicable, since toxic levels are at or below therapeutic levels (F S Barr, personal communication)

Tennecetin was given intravenously to rats, 100 U/kg of body weight Blood was withdrawn and assayed When the blood was centrifuged and the serum assayed no tennecetin activity could be detected However, when the red blood cells were hydrolyzed, the tennecetin activity reappeared

Blood samples withdrawn one hour later were treated in the same manner, but no tennecetin activity was found The urine collected for the same period did not display any antibiotic activity These tests seem to indicate that the tennecetin was either broken down or deposited in some tissue (F S Barr, personal communication)

Tennecetin has been tested for toxicity by the oral route in laboratory animals and in human beings The results of these tests indicate the antibiotic is of low toxicity when so employed (F S Barr personal communication)

Topical in Vivo Studies Reported by Others

V J Derbes (personal communication to the S F Massengill Co) treated 23 patients who had tinea capitis and 12 who had tinea corporis and cruris A summary of his report follows

Tinea capitis	Cases	Cures	Treatment (in months)	
			1	2
M audouini	16	6	11	5
T tonsurans	6	1	5	1
Kerion	1	1	1	

Four of the 6 cures in *M audouini* infections occurred in patients treated for 2 months The only cure in the *T tonsurans* group was obtained after 2 months of treatment

Tinea corporis and Tinea cruris	Cases	Cures	Treatment (in months)	
			1	2
T rubrum	10	0	9	1
T tonsurans	1	1	1	
Erythrasma	1	1	1	

I can say only that treatment in this group of cases was of too short duration to expect reasonably satisfactory response and does not represent adequate appraisal of this antibiotic agent in this group of patients Additionally, the 350 U gm ointment was used in all but 2 cases (in these the 500-U ointment was used)

R M Metcalfe (personal *communication to the S E Massengill Co*) reported on the use of tennecetin ointment (350 U/gm) in 7 patients as follows

Diagnosis	Result	Duration
Tinea corporis	Good	5 days
Tinea corporis	Good	10 days
Tinea corporis	Excellent	7 days
Tinea pedis	Excellent	7 days
Tinea pedis	Good	14 days
Tinea cruris	Fair	10 days
Onychomycosis	Poor	2 to 3 months

In summary this investigator reports excellent results in 2 good results in 3 and improvement in the fifth patient with dermatomycoses of the glaborous skin There was no response in the patient with onychomycosis In this group treatment was carried out for only short intervals

L Goldman (personal communication) reported on the use of tennecetin ointment (500 U/gm) in 17 patients Six had tinea pedis (3 proved by culture to be due to *T mentagrophytes*), 1 had *tinea corporis (proved by culture* to be due to *T gypseum*) 2 had tinea cruris 3 had tinea versicolor, and 5 had moniliasis of the glaborous skin (proved by culture) Of the tinea pedis group *1 patient had an excellent result* and 2 had fair results One of the remaining 3 flared and the other 2 had poor response to the agent after 3 to 8 weeks of treatment The patient who had tinea corporis did not respond after 3 weeks of treatment One of the patients who had tinea cruris cleared the other flared after 3 weeks of treatment The 3 patients who had tinea versicolor all cleared in 1 to 3 weeks while using tennecetin ointment but all relapsed when this agent was discontinued Only 1 of the 5 patients with moniliasis had a satisfactory response after use of the antibiotic for from 3 to 6 weeks In *this group the treatment interval was short*

R Greenblatt (personal communication to the S E Massengill Co) reported on 9 patients who had vaginal moniliasis (all proved by culture) Seven patients cleared completely and had *3 negative cultures 3 weeks after treatment* was discontinued These patients were treated for 12 to 24 days Two *patients each treated* for 12 days had positive cultures after 3 weeks although they had some amelioration of symptoms and marked relief of pruritus *One* tennecetin suppository (400 U) was inserted into the vagina nightly In this *group of patients 77 7 per cent* responded to this tennecetin preparation

T C Todd (personal communication to the S E Massengill Co) treated 19 patients with moniliasis involving the vulva (all proved by culture) with *tennecetin vaginal jelly (400 U/gm)* one half to 1 applicatorfull in the vagina once or twice daily or one tennecetin suppository (400 U) in the vagina daily He reported good response in 9 patients improvement in 9 patients and a poor result in *1 patient*

F H Payne (personal communication to the S E Massengill Co) treated 6 patients who had moniliasis with tennecetin vaginal suppositories (400 U) for from 6 to 24 days *The diagnosis was* in all

patients before treatment Three patients had excellent results, 2 good results, and 1 a poor result All but 1 had negative smears after treatment

H L Riva and R E Staats (personal communication to the Massengill Co) treated 10 patients who had moniliasis and 13 who had trichomoniasis of the vagina They prescribed the insertion into the vagina of 1 tennecetin (400 U) vaginal suppository twice daily The night insertion was preceded by a vinegar douche Eight of the patients who had moniliasis became asymptomatic and had negative smears after treatment The other 2 patients of this group became asymptomatic, but continued to have positive smears Eight of the patients who had trichomoniasis became asymptomatic following treatment, but in only 2 was negative microscopic evidence of trichomonal infection obtained The remaining 5 patients who had trichomoniasis were unimproved symptomatically and continued to have microscopic evidence of trichomoniasis after treatment

TABLE 4
DERMATOPHYTOSES TREATED

Diagnosis	No cases
Tinea pedis	84
Tinea mani	25
Tinea corporis	48
Tinea capitis	30
Tinea cruris	23
Tinea versicolor	8
Onychomycosis	21
Blastomycetia interdigitalis	6
Total	245

Topical in Vitro Studies Conducted in Our Office

We attempted on evaluation of the therapeutic efficiency of tennecetin broad spectrum antifungal antibiotic when applied topically for the treatment of selected dermatoses

All patients (drawn from private practice) were instructed to apply the ointment to a small area watch for and report any untoward reaction such as increased irritation, erythema, extension of eruption, and the like, before application was made to large areas If untoward reaction did occur, patients were instructed to stop use immediately and return for observation Applications of ointments were prescribed form 1 to 3 (occasionally 4) times daily Usually ointments were applied twice daily Frequency of application depended upon the condition All patients were observed at weekly intervals This report is based on a study period of approximately 23 months

In the early part of the study concentrations of tennecetin of 70 and 140 U gm were employed later concentrations of tennecetin 200 and 350 U /gm were used Finally, in the last few months formulation of tennecetin ointment studied contained 500 U gm As the study progressed the concentration of tennecetin in the ointment base was gradually increased as it became apparent that the early weaker concentrations did not give clinical results consistent

with *in vitro* studies As the concentration of tennecetin in the ointment base was increased, the response index was increased The tennecetin was incorpo-

TABLE 5
MONILIASIS TREATED

Location	No cases
Vulva	17
Mouth	10
Commissures	2
Paronychia	5
Nail	1
Total	35

TABLE 6
DURATION TREATMENT IN MONTHS

1 3	4 5	6-12
170	77	33

TABLE 7
PATIENTS TREATED WITH 500 U TENNECETIN OINTMENT

Diagnosis	No cases
Tinea pedis	17
Tinea mani	4
Tinea corporis	10
Tinea capitis	10
Tinea cruris	8
Tinea versicolor	6
Blastomycetia interdigitalis	4
Moniliasis	8
Total	67

TABLE 8
RESULTS WITH 500 U TENNECETIN OINTMENT

	No cases	Per cent
Excellent	30	44 7
Good	16	23 5
Fair	11	16 4
Poor	10	14 9

rated in an ointment base consisting of beeswax, white petrolatum, and liquid petrolatum

Tennecetin ointments of various concentrations were used topically in 280 patients It seems irrelevant to record here the results of studies with the

weaker concentrations of tennecetin ointment, therefore, only the results with the 500 U/gm ointment are tabulated hereafter.

Diagnoses in all cases were confirmed by KOH preparation, or culture, or both. TABLE 4 demonstrates the various types of dermatophytoses and the number of cases treated with the several concentrations of tennecetin ointment. These dermatoses had persisted in these patients for from 1 month to 20 years.

TABLE 5 illustrates the location of involvement and the number of cases of moniliasis treated with the several concentrations of tennecetin formulations.

TABLE 6 indicates the duration of treatment in months.

TABLE 7 depicts the various types of conditions and the number of cases treated with the 500 U/gm tennecetin ointment.

TABLE 8 records the clinical results obtained with the 500 U tennecetin formulation in 67 patients.

TABLE 9 lists the reactions observed in the 280 patients studied employing the various concentrations of tennecetin in either ointment or jelly base. Sensitization was not observed.

TABLE 9

REACTIONS OBSERVED IN 280 PATIENTS

Irritating	6
Desquamating fissuring	2
Burning	2
Sensitizing	0
Total	10

Discussion of Therapeutic Results

It is now apparent that a concentration of at least 500 U of tennecetin per gm of ointment base is requisite for reasonable therapeutic response in the topical therapy of the various superficial fungal infections studied, and in moniliasis.

The response with the 500 U tennecetin formulation was considered excellent in 44.7 per cent of the cases studied and good in an additional 23.5 per cent. This appears to be a reasonable therapeutic response index.

Of the 280 patients studied, only 10 showed reaction of any type. Six demonstrated irritation; 5 of these had tinea cruris with superimposed contact factors. One had tinea corporis on the flexor surface of the left forearm that was already eczematized from previous medication. Two patients desquamated (in spite of the lubricating nature of the ointment base) to the point of fissuring. Two reported a burning sensation with each application of the ointment lasting for such long intervals that it was necessary to discontinue the ointment. Objectively, these 2 patients did not show irritation or eczematization.

Patients with tinea versicolor responded regularly to treatment with the 500 U/gm tennecetin ointment. However, relapses were fairly regular when treatment was discontinued after clinical and laboratory data were negative. This appeared to be definitely due to reinfection from contaminated fomites. In 5 patients who have, after clearing, continued to use the tennecetin 500

U/gm ointment weekly, as a preventive measure, there has been no recurrence in the 6 months this procedure has been employed

As was to be expected, in moniliasis of the female genitalia, it was necessary to eradicate vaginal moniliasis, if present, by use of tennecetin jelly or suppositories in order to obviate recurrence on the mucosal and cutaneous parts of the genitalia and adjacent areas. Patients with moniliasis experienced an early relief of pruritus followed by desquamation of the pseudomembrane on mucosal surfaces and scaling on cutaneous surfaces

Patients who had onychomycosis or moniliasis of the nails failed to respond even to prolonged use of the tennecetin ointments in the concentrations employed

Summary

The general characteristics of *S. chattanoogensis* and the antibiotic—designated as tennecetin, a tetraene—derived from it, have been summarized. The chemical, physical, and biological properties, toxicological data, *in vitro* sensitivity data, and available systemic *in vivo* studies of tennecetin have been partially reviewed. Clinical studies with topical tennecetin ointment, vaginal jelly, and vaginal suppositories conducted by others and those done personally have been presented

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To date, results of studies of the broad spectrum, antifungal antibiotic tennecetin for the topical treatment of superficial fungal infections appear encouraging

It is my opinion that a better therapeutic response index might be obtained if the tennecetin were used in formulations employing a vanishing lotion or ointment base, or both. Such formulations should allow better penetration of the agent into the tissues

If formulations can be developed that will allow this agent to exert its full antifungal activity when employed topically, it should eventuate as a very effective agent for the topical therapy of superficial fungal infections

References

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ANALYSIS OF AMPHOTERICIN TREATMENT FAILURES IN SYSTEMIC FUNGAL DISEASE

John P Utz and Vincent T Andriole

National Institute of Allergy and Infectious Diseases Public Health Service Bethesda Md

Administered intravenously, amphotericin has been an effective treatment in a number of patients with cryptococcosis, histoplasmosis, coccidioidomycosis, and blastomycosis [1-4] In addition, isolated cases of aspergillosis,[3] sporotrichiosis,[3] and *Candida* infections of the peritoneum[7] and pleural cavity[8] have been treated successfully by intravenous or local (intraperitoneal or intrathoracic) therapy Such success is a significant advance in the treatment of these diseases

At the National Institutes of Health, we have studied 40 patients with 41 culturally confirmed systemic fungal infections treated intravenously with amphotericin The results of this treatment are shown in TABLE 1 The details of treatment have been described previously in most cases,[8,9] and the patient numbers refer to these and forthcoming reports The success achieved in treatment of 20 of these infections has led to the present analysis of the 20 infections that represent treatment failures (the remaining patient is currently on therapy) It becomes apparent that a number of factors played a part in these failures

Early Discontinuation of Treatment

Patient 11 has had known pulmonary blastomycosis since March 1955 In May 1957 she received amphotericin over a period of 11 days, but discontinued treatment after a total dose of only 110 mg She is asymptomatic, but sputum cultures have been persistently positive except for 2 months immediately following treatment

Patient 6, with known cryptococcal meningitis since January 1956, received amphotericin in July 1957 for 13 days in total dosage of 223 mg and then broke off treatment

Such obvious causes for treatment failure are worthy of mention only to emphasize the fact that the side effects of intravenous therapy (chills, fever, nausea, vomiting, anorexia) may be more distressing to a patient than the symptoms of his disease

Advanced Disease

In contrast to the acute disease encountered with most bacterial and viral infections, systemic fungal diseases are generally chronic In a few instances, however, the total course of fungal illness may be a matter of a month or less, leaving little time for treatment after diagnosis has been made

Patient 3, a 6-month old infant with extensive disseminated histoplasmosis, lived only 24 days after hospitalization and only 7 days after institution of intravenous therapy with a total dose of 30 mg of amphotericin

Patient 34 had had correction of a mitral valve insufficiency by open heart surgery on November 6, 1958 On return admission for postoperative evalua-

U/gm ointment weekly, as a preventive measure, there has been no recurrence in the 6 months this procedure has been employed

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tion, February 18, 1959, she gave a history of intermittent fever and increased dyspnea of about 3 weeks' duration. Six blood cultures on February 18, 19, and 20 were positive for *Candida guillermondii*. On February 21 amphotericin treatment was instituted, but on February 24 the patient expired after receiving only 140 mg. of drug. The fulminating course of disease in this patient precluded a long course of therapy, although we have not found amphotericin effective in other cases of *Candida* endocarditis.

Patient 17 with cryptococcal meningitis survived for only 27 days after onset of disease and died after receiving only 621 mg. of amphotericin.

Insufficient Amount of Drug

Patient 33, a 23-year-old Negro man, while in the Air Force and stationed in Arizona as a construction worker, had a pulmonary disease diagnosed as coccidioidomycosis from which he slowly improved. However, his chest film,

TABLE 1
TREATMENT WITH AMPHOTERICIN INTRAVENOUSLY AND INTRATHECALLY

Disease	Apparent recovery	Improved	On treatment	Relapse	Unchanged	Dead	Total
Histoplasmosis	4*	3	0	1	0	2	10
Cryptococcosis	4	3*	0	1	1	3	12
Blastomycosis	4*	0	0	1	1	0	6
Coccidioidomycosis	0	1	1	1	2	1	6
Candidiasis	1	0	0	0	0	5	6
Maduramycosis	0	0	0	0	1	0	1
Totals	13	7	1	4	5	11	41†
					20		

* One patient dead of other cause
† Dual infection (cryptococcosis and maduramycosis) in one patient

at time of discharge, September 1957, showed a 2 cm. nodular density at the superior pole of the right hilus. In November 1958 he began to have headache and, following studies that indicated possible posterior fossal tumor, underwent craniotomy. A multilobed cystic mass containing pus was removed from the right vermis of the cerebellum. Cultures of this, cerebrospinal fluid, and sputum were positive for *Coccidioides*. Cultures became negative, and there was clinical improvement during a 4-week course of treatment with 800 mg. of amphotericin. The patient was discharged April 15, 1959, but was readmitted May 15 with lymphocytic meningitis due to *Coccidioides*. He expired June 4 after unsuccessful therapy with another antifungal drug. Although, as will be observed later, success with amphotericin in coccidioidomycosis is limited, in retrospect it seems that this patient received less than optimal dosage.

Although amphotericin treatment of blastomycosis has been generally successful, patient 19 relapsed 8 months after a 3-month course of 2298 mg. of amphotericin. His infection had been severe, with osteomyelitis of lumbar spines 1, 2, 3, 4 and psoas abscess. No pulmonary lesion had been seen on chest film, but sputum specimens had been culturally positive. He responded

well to surgical drainage of the psoas abscess and the amphotericin treatment, but then relapsed The possibility that treatment failure was related to insufficient total dosage is suggested by the successful treatment of patient 12 who had equivalent, if not more extensive, disease and who responded without relapse to a course of treatment over a 7-month period with a dosage (5185 mg) more than twice that of patient 19

Patient 63 is of especial interest because he had unusual skin manifestations of cryptococcal infection but normal cerebrospinal fluid at the time of his initial hospitalization [10] From September 3 to October 6, 1956, 1 gm of amphotericin was given, and on rehospitalization, December 5 to December 24, 1956, an additional course of 880 mg was given A third course of treatment (430 mg) was administered from July 5, 1957 to January 1, 1958, following which the skin lesions healed and the patient returned to work On August 18, 1959, he was readmitted, and for the first time symptoms, cerebrospinal fluid findings, and cultures indicated cryptococcal meningitis He was restarted on amphotericin treatment, transferred to the Clinical Center, and treated over a 2 month period with 3140 mg of drug Normal cerebrospinal fluid findings were present at end of therapy, November 10, 1959

Death From Associated Disease

Patient 27 had had disseminated histoplasmosis dating to November 1957 At the time of admission October 22, 1958, he had positive cultures of blood, bone marrow, urine and oral ulcer Despite this severe disease, he seemed to be making a satisfactory recovery with negative cultures after 2 weeks' treatment with 460 mg of amphotericin Despite normal steroid values in blood and urine, adrenal insufficiency had been suspected on the basis of hypotension, hyponatremia, and hyperkalemia, and the patient had been on 10 mg of hydrocortisol daily On December 3 the patient developed acute pyelonephritis, subsequently shown to be due to staphylococcus, which seemed to respond to penicillin, tetracycline, and steroids (increased to 50 mg /day) On December 10 he suddenly developed signs of pneumonia and died within 24 hours despite additional antistaphylococcal therapy Autopsy revealed staphylococcal bronchopneumonia and caseous lesions of the adrenal glands Cultures of urine, blood, ascitic fluid, spleen, kidneys, liver, lungs, alveolar ridge and lymph nodes were negative for *Histoplasma* However, cultures of both adrenal glands were positive In this case treatment failure seems to be the direct result of staphylococcal pneumonia occurring in a patient recovering from, but with crucial residual adrenal disease due to, *Histoplasma*

Patient 44 similarly seemed to be responding to a course of 457 mg of amphotericin for cryptococcal meningitis Fever persisted, however, and he died suddenly of *Pseudomonas septicemia*

Relapse with Pregnancy

Patient 21, a 25 year old Negro woman, had known coccidioidomycosis since July 1957, manifested by subcutaneous abscesses of the left shoulder, supraclavicular fossa, groin, thoracic paravertebral area, and thigh, septic arthritis of the right metacarpophalangeal joint and left knee, osteomyelitis of the thoracic spines 10, 11, 12, pubis, left fifth proximal phalanx, clavicle, sternum, and tibia,

neumonic infiltrate of the right upper lobe, and empyema Cultures of iultiple sites were positive for *Coccidioides* After many surgical incisions ith drainage and a 6 months' course of amphotericin treatment (7300 mg otal) ending March 2, 1959, she seemed to improve In June 1959 at a return isit remarkable clinical improvement had continued and cultures of gastric uid—the only readily available specimen—were negative Subsequent to us she became pregnant (last menstrual period late June 1959) and in Septem er 1959 noted onset of a subcutaneous paravertebral mass from which, on hospitalization in November 1959, *Coccidioides* was again cultured Preg ancy has been known for some time to have a deleterious effect on the course f coccidioidomycosis, and this patient represents relapse after prior treatment nd improvement

Relapse Associated with Steroid Treatment

A 62 year-old farmer (patient 28) in July 1958 developed an illness char terized by fever, fatigue, mental changes, and a lymphocytic meningitis le was admitted to the Clinical Center in December 1958, and *Histoplasma ipsulatum* was isolated from multiple cerebrospinal fluid specimens Cultures ecame negative, and the patient showed marked improvement after 2621 mg mphotericin treatment administered from December 30, 1958 to March 31, 959 Hospitalization was complicated by concomitant diabetes mellitus, yelonephritis, thrombophlebitis, pulmonary embolus, and uveitis For the tter, the patient received local therapy with atropine and cortisol beginning lay 12, 1959 Uveitis did not improve, however, and on June 11, 1959, sys mic therapy with prednisone was begun in dose of 30 mg /day Clinical lapse of histoplasmosis began about June 29, and cerebrospinal fluid cultures ere positive July 5 Following treatment with 2589 mg additional ampho ricin ending September 24, the patient made an apparent recovery and was ischarged October 29

Essentially Resistant Disease

Of all fungal diseases treated with amphotericin in this series, the most re stant as a group have been fixed *Candida* infections Four cases of ndocarditis and 1 case of *Candida* meningitis have failed to respond to treat ient, and all patients have perished from their disease

One of these 5, patient 34, has already been discussed

It is of particular interest that only 2 (patients 23 and 45) of these 4 remain ig patients were infected with an organism that *in vitro* was resistant to a level f drug greater than that found with "sensitive" strains of fungi or greater than level of amphotericin achievable in serum of treated patients Patient 23 eceived 831 mg of amphotericin during a 4 week course of treatment and pa ient 45 1644 mg during a 9 week course

The remaining 2 patients (22 and 24) received 2136 mg during a 48 week eriod and 2370 mg during a 5 month period, respectively Serum levels were reached in excess of (by factors of 4 and 7 times) the *in vitro* sensitivity (0 04 and 0 07 μg /ml) of the infecting strains In all 4 patients, however, blood or cerebrospinal fluid cultures remained positive during life and at autopsy In the 4 cases of endocarditis, 3 had large vegetations on valves and endocardium

measuring, in 2 cases, (patient 23) 8 cm and (patient 45) 2 cm The size of these vegetations suggests that organisms were multiplying in depths of fibrin that may not have been penetrated by the antibiotic This plausible explanation of persistence of organisms would not seem to account, however, for the large colony counts circulating in the blood stream, apparently not suppressed or killed by serum antimicrobic levels

Multiple infection by 2 fungal agents was seen in patient 31, who had cryptococcal meningitis and a *Madurella* cellulitis of the left leg Amphotericin in total dose of 1551 mg intravenously and 9 5 mg intrathecally during a 7 week period was sufficient to convert cerebrospinal fluid cultures to negative and produce marked improvement in cerebrospinal fluid findings No appreciable change was observed, however, in the cultures or appearance of the lesion due to *Madurella*

Patient 30 had meningitis due to a strain of *Cryptococcus* resistant *in vitro* to 2 to 4 times the amount of amphotericin that suppressed strains from other patients He died of his disease despite over 3800 mg of amphotericin intravenously and 15 mg intrathecally (0 5 mg, 2 to 3 times a week) over a 4 month period

Indeterminate Factors

Factors are not known that may have played a part in treatment failures in 2 cases (patients 14 and 69) who had pulmonary coccidioidomycosis Large total dosages of drug (2600 and 3525 mg) were administered for long periods (11 weeks and 4 months, respectively), and in patient 14 a good serum level was proved on at least 1 occasion Steroids or other disease did not play a part and the sensitivities of the organisms were not determined

These 2 patients, as well as the 6 who had "essentially resistant disease" (see above) compose that group (8 of 40 patients, 20 per cent) who failed completely to respond to otherwise adquate amounts of drug given for a significant period of time

Summary

Unprecedented success has been achieved with amphotericin treatment of many cases of blastomycosis, histoplasmosis, cryptococcosis coccidioidomycosis, and in isolated cases of aspergillosis, sporotrichosis, and some types of *Candida* infections This success suggested an evaluation of 20 amphotericin treatment failures in the 41 infections (40 patients) studied at the Clinical Center of the National Institutes of Health Factors that seemed important in these failures included early discontinuation of treatment, advanced disease, insufficient amount of drug, death from associated disease, relapse with pregnancy, relapse associated with steroid treatment, essentially resistant infection, *and indeterminate factors*

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